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PATENT APPLICATION

NUCLEAR RECEPTOR LIGANDS AND LIGAND BINDING DOMAINS

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NUCLEAR RECEPTOR LIGANDS AND LIGAND BINDING DOMAINS

ACKNOWLEDGMENTS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the following provisional applications: United States Ser. No. 60/008,540 and 60/008,543, filed December 13, 1995, and Ser. No. 60/008,606, filed December 14, 1995. This application claims the benefit of the following U.S. patent application: United States Ser. No. 08/764,870, filed December 13, 1996.

INTRODUCTION

Technical Field

This invention relates to computational methods for designing ligands that bind to nuclear receptors, crystals of nuclear receptors, synthetic ligands of nuclear receptors and methods of using synthetic ligands.

Background

Nuclear receptors represent a superfamily of proteins that specifically bind a physiologically relevant small molecule, such as hormone or vitamin. As a result of a molecule binding to a nuclear receptor, the nuclear receptor changes the ability of a cell to transcribe DNA, i.e. nuclear receptors modulate the transcription of DNA, although they may have transcription independent actions. Unlike integral membrane receptors and membrane associated receptors, the nuclear receptors reside in either the cytoplasm or nucleus of eukaryotic cells. Thus, nuclear receptors comprise a class of intracellular, soluble ligand-regulated transcription factors.

Nuclear receptors include receptors for glucocorticoids (GRs), androgens (ARs), mineralocorticoids (MRs), progestins (PRs), estrogens (ERs), thyroid hormones (TRs), vitamin D (VDRs), retinoids (RARs and RXRs), peroxisomes (XPARs and PPARs) and icosanoids (IRs). The so called "orphan receptors" are also part of the nuclear receptor superfamily, as they are structurally homologous to the classic nuclear receptors, such as steroid and thyroid receptors. To date, ligands have not been identified with orphan receptors but it is likely that small molecule ligands will be discovered in the near future for this class of transcription factors. Generally, nuclear receptors specifically bind physiologically relevant small molecules with high affinity and apparent Kd's are commonly in the 0.01 - 20 nM range, depending on the nuclear receptor/ligand pair.

Development of synthetic ligands that specifically bind to nuclear receptors has been largely guided by the trial and error method of drug design despite the importance of nuclear receptors in a myriad of physiological processes and medical conditions such as hypertension, inflammation, hormone dependent cancers (e.g. breast and prostate cancer), modulation of reproductive organ function, hyperthyroidism, hypercholesterolemia and obesity. Previously, new ligands specific for nuclear receptors were discovered in the absence of information on the three dimensional structure of a nuclear receptor with a bound ligand. Before the present invention, researchers were essentially discovering nuclear receptor ligands by probing in the dark and without the ability to visualize how the amino acids of a nuclear receptor held a ligand in its grasp.

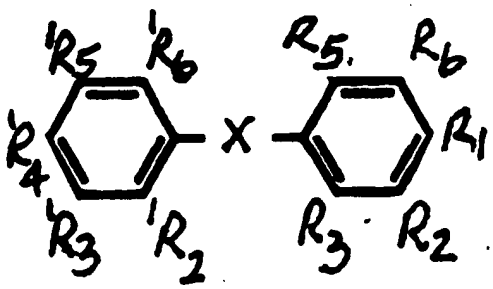
Consequently, it would be advantageous to devise methods and compositions for reducing the time required to discover ligands to nuclear receptors, synthesize such compounds and administer such compounds to organisms to modulate physiological processes regulated by nuclear receptors.

SUMMARY OF THE INVENTION

The present invention provides for crystals of nuclear receptor ligand binding domains with a ligand bound to the ligand binding domain (LBD). The crystals of the present invention provide excellent atomic resolution of the amino acids that interact with nuclear receptor ligand, especially thyroid receptor ligands. The three dimensional model of a nuclear receptor LBD with a ligand bound reveals a previously unknown structure for nuclear receptors and shows that the ligand is bound in a water inaccessible binding cavity of the ligand binding domain of the nuclear receptor.

The present invention also provides for computational methods using three dimensional models of nuclear receptors that are based on crystals of nuclear receptor LBDs. Generally, the computational method of designing a nuclear receptor ligand determines which amino acid or amino acids of a nuclear receptor LBD interact with a chemical moiety (at least one) of the ligand using a three dimensional model of a crystallized protein comprising a nuclear receptor LBD with a bound ligand, and selecting a chemical modification (at least one) of the chemical moiety to produce a second chemical moiety with a structure that either decreases or increases an interaction between the interacting amino acid and the second chemical moiety compared to the interaction between the interacting amino acid and the corresponding chemical moiety on the natural hormone.

Also provided is a method of modulating the activity of a nuclear receptor. The method can be *in vitro* or *in vivo*. The method comprises administering *in vitro* or *in vivo* a sufficient amount of a compound of the following formula:



FORMULA I.

where the compound fits specially and preferentially into a nuclear hormone receptor LBD of interest. The method is exemplified by modulating the activity of a thyroid receptor (TR).

For modulating TR activity, a compound of Formula I is employed that fits spacially and preferentially into a TR ligand binding domain (TR LBD), including compounds specific for a TR LBD isoform of interest. Of particular interest are the TR LBD isoforms α (TR-α) and β (TR-β). Additional compounds of interest include derivatives of Formula I, such as those compounds having the biphenyl (ϕ-X-ϕ) or single phenyl (ϕ-X or X-ϕ) nucleus of Formula I and its corresponding substituent groups described herein. Compounds that are interatively designed using structural information gleaned from these compounds and which modulate nuclear hormone receptor activity also are of interest.

The present invention also includes a method for identifying a compound capable of selectively modulating the activity of a nuclear receptor. This aspect of the invention is exemplified by a method for identifying a compound capable of selectively modulating the activity of a TR isoform. The method comprises modeling test compounds that fit spacially and preferentially into a TR LBD isoform of interest using an atomic structural model of a TR LBD isoform bound to a test compound, screening the test compounds in a biological assay for TR isoform activity characterized by binding of a test compound to a TR LBD isoform, and identifying a test compound that selectively modulates the activity of a TR isoform. The compounds may be those of Formula I or derivatives thereof, including compounds having a biphenyl or single phenyl nucleus of Formula I.

Further included is a method for identifying agonist or antagonist ligands of a nuclear receptor using the atomic coordinates of a LBD in conjunction with a computerized modeling system. This aspect of the invention is exemplified by identifying a TR agonist or antagonist ligand by providing the atomic coordinates of a TR LBD to a computerized modeling system, modeling ligands which fit spacially into the TR LBD, and identifying in a biological assay for TR activity a ligand which increases or decreases TR activity. The compounds can be those of Formula I or derivatives thereof, including compounds having a biphenyl or single phenyl nucleus of Formula I.

Also provided is a method of identifying a compound that selectively modulates the activity of one type of nuclear receptor compared to other nuclear hormone receptors. The method is exemplified by modeling test compounds which fit spacially into a TR LBD using an atomic structural model of a TR LBD, selecting a compound comprising conformationally constrained structural features that interact with conformationally constrained residues of a

TR LBD, and identifying in a biological assay for TR activity a compound that selectively binds to a TR LBD compared to other nuclear receptors. The conformationally constrained features involved in receptor-selective ligand binding can be identified by comparing atomic models of receptor isoforms bound to the same and/or different ligands. The methods facilitate design and selection of compounds that have increased selectivity for a particular nuclear receptor. The compounds may be those of Formula I or derivatives thereof, including compounds having the biphenyl or single phenyl nucleus of Formula I.

Another aspect of the invention is a method for increasing the receptor selectivity of a compound for a particular type of nuclear receptor. This involves the chemical modification of a substituent group of a compound of Formula I to generate compounds which have increased selectivity for one type of receptor. For example, chemical modification of a substituent group of the compound of Formula I can be used to introduce additional constraints into a compound that modulates TR activity to increase its selectivity *in vivo* for TR-type receptors. Additional constraints also may be added for stability. The modified groups will preferably interact with a conformationally constrained structural feature of a TR LBD that is conserved among TR isoforms. A more preferred method comprises selecting compounds having conformationally constrained groups that interact with conformationally constrained residues of a TR LBD conserved among TR isoforms. The compounds can be those of Formula I or derivatives thereof, including compounds having the biphenyl or single phenyl nucleus of Formula I.

The invention finds use in the selection and characterization of peptide, peptidomimetic or synthetic compounds identified by the methods of the invention, particularly new lead compounds useful in treating disorders related to nuclear receptor-based deficiencies, including TR-related disorders. For TR-related disorders, the compounds and methods of the invention can be used to modulate TR activity by administering to a mammal in need thereof a sufficient amount of compound of Formula I or derivative thereof that fits spacially and preferentially into a TR LBD.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram illustrating computational methods for designing ligands that interact with nuclear receptors of the nuclear receptor superfamily.

FIG. 2 is a schematic representation of nuclear receptor structures, indicating regions of homology within family members and functions of the various domains.

FIG. 3 shows the aligned amino acid sequences of the ligand binding domains of several members of the nuclear receptor superfamily.

FIG. 4 is a ribbon drawing of the rat TR-α LBD with secondary structure elements labelled. The ligand (magenta) is depicted as a space-filling model. Alpha helices and coil conformations are yellow, beta strands are blue.

FIG. 5 shows two cross-sections of a space-filling model of rat TR-α exposing the ligand (magenta) tightly packed within the receptor.

FIG. 6 is a schematic of the ligand binding cavity. Residues which interact with the ligand appear approximately at the site of interaction. Hydrogen bonds are shown as dashed lines between the bonding partners; distances for each bond are listed. Non-bonded contacts are shown as radial spokes which face toward interacting atoms.

FIG. 7 is the distribution of crystallographic temperature factors in the refined rat TR-α LBD. The distribution is represented as a color gradation ranging from less than 15 (dark blue) to greater than 35 (yellow-green).

FIG. 8 is a ribbon drawing of the rat TR-α LBD showing the c-terminal activation domain to ligand. Residues which comprise the c-terminal activation domain (Pro393-Phe405) are depicted as a stick representation. Hydrophobic residues, particularly Phe401 and Phe405 (blue) face inwards toward the ligand. Glu403 (red) projects outward into the solvent.

FIG. 9 is an electrostatic potential surface of the rat TR-α LBD, calculated using GRAPH. Negative electrostatic potential is red; positive electrostatic potential is blue. The c-terminal activation domain forms a largely hydrophobic (white). The Glu403 is presented as a singular patch of negative charge (red).

FIG. 10 is a diagram comparing agonists and antagonists for several nuclear receptors.

FIG. 11 is the synthetic scheme for preparation of TS1, TS2, TS3, TS4 and TS5.

FIG. 12 is the synthetic scheme for preparation of TS6 and TS7.

FIG. 13 is the synthetic scheme for preparation of TS8.

FIG. 14 is the synthetic scheme for preparation of TS10.

FIG. 15 depicts the chemical structures of several TR ligands.

FIG. 16 is a graph illustrating competition assays in which T_3 and Triac compete with labeled T_3 for binding to human TR-α or human TR-β.

FIG. 17 depicts a Scatchard analysis of labelled T_3 binding to TR-α and TR-β.

FIG. 18 is a chart showing the effect of TS-10 on the transcriptional regulation of the DR4-ALP reporter gene in the presence or absence of T3 as assayed in TRAFα1 reporter cells.

FIG. 19 is a chart showing the effect of TS-10 on the transcriptional regulation of the DR4-ALP reporter gene in the presence or absence of T3 as assayed in TRAFβ1 reporter cells.

FIG. 20 is a chart showing the effect of TS-10 on the transcriptional regulation of the DR4-ALP reporter gene in the presence or absence of T3 as assayed in HepG2, a liver reporter cell line.

FIG. 21 is a partial ribbon drawing of TR-α LBD with T3 in the ligand binding cavity. Selected interacting amino acids are labelled, including Ile221, Ile222 and Ser260, Ala263, Ile299 and Leu 276.

FIG. 22 is a partial ribbon drawing of TR-α LBD with T3 and Dimit superimposed in the ligand binding cavity. Interactions with Ile221, Ile222, Ala260, Ile 299 and Leu276 are labelled.

FIG. 23 is a partial ribbon drawing of TR-α LBD with T3, illustrating the three Arginine residues (Arg228, Arg262 and Arg 266 (dark stick figures)) of the polar pocket, three water molecules HOH502, HOH503 and HOH504, with hydrogen bonds indicated by dotted lines.

FIG. 24 is a partial ribbon drawing of TR-α LBD with Triac, illustrating the three Arginine residues (dark stick figures) of the polar pocket, water molecules (HOH503, HOH504 and HOH600), with hydrogen bonds indicated by dotted lines.

FIG. 25 is a partial ribbon drawing of the TR-α LBD with T3 and Triac superimposed in the ligand binding cavity. The drawing shows several interacting amino acid residues in the polar pocket that remain unchanged whether T3 or Triac occupies the ligand binding cavity: Arg262, Asn179, HOH503 and HOH504, and Ser277. Both Arg228 and Arg 266 occupy two different positions, depending on whether T3 or Triac is bound.

FIG. 26A and 26B are stereochemical representations of the TR-α LBD with Dimit bound.

FIG. 27 is a partial ribbon drawing of TR-β LBD with GC-1 in the ligand binding cavity. Amino acids Arg282, Arg316, Arg320, Asn 331 and His435 are labelled.

FIG. 28 is a partial ribbon drawing of TR-β LBD with Triac in the ligand binding cavity. Amino acids Arg282, Arg316, Arg320, Asn331 and His435 are labelled.

FIG. 29 is a partial ribbon drawing of TR-βLBD with GC-1 (Blue) overlayed with TR-α LBD with Dimit (Red) in the ligand binding cavities. Amino acids Arg228, Arg262, Arg266 and Ser277 (TR-α LBD), and Arg282, Arg316, Arg320 and Asn331 (TR-β LBD) are labelled.

FIG. 30 is a partial ribbon drawing of TR-β LBD with Triac (Blue) overlayed with TR-α LBD with Triac (Red) in the ligand binding cavities. Amino acids Arg228, Arg262, Arg266, Ser277 and His381 (TR-α LBD), and Arg282, Arg316, Arg320 and His435 (TR-β LBD) are labelled.

FIG. 31 is a graph showing competition curves comparing wildtype TR-α and TR-β to a variant TR-β having a single amino acid substitution in the ligand binding domain.

FIG. 32 shows atomic numbering for thyronine-like ligands.

APPENDIX 1 is an appendix of references.

APPENDIX 2 is a chart of amino acids that interact with a TR ligand, for TR complexed with Dimit, Triac, IpBr2, T3 and GC-1.

APPENDIX 3 is a chart of atomic coordinates for the crystal of rat TR-α LBD complexed with Dimit.

APPENDIX 4 is a chart of atomic coordinates for the crystal of rat TR-α LBD complexed with Triac.

APPENDIX 5 is a chart of atomic coordinates for the crystal of rat TR-α LBD complexed with $IpBr_2$.

APPENDIX 6 is a chart of atomic coordinates for the crystal of rat TR-α LBD complexed with T_3.

APPENDIX 7 is a chart of atomic coordinates for the crystal of human TR-β LBD complexed with Triac.

APPENDIX 8 is a chart of atomic coordinates for the crystal of human TR-β-LBD complexed with GC-1.

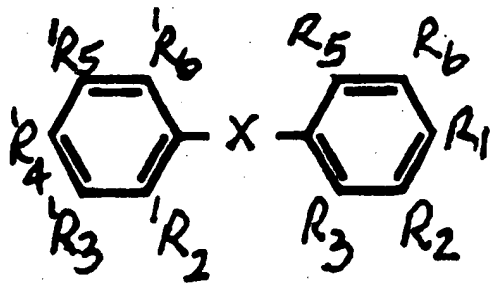
DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

The present invention provides new methods, particularly computational methods, and compositions for the generation of nuclear receptor synthetic ligands based on the three dimensional structure of nuclear receptors, particularly the thyroid receptor (herein referred to as "TR"). Previously, the lack of three dimensional structural information about the ligand binding domain of a nuclear receptor thwarted the field of nuclear receptor drug discovery, especially the absence of three dimensional structural information relating to a nuclear receptor with a ligand bound.

Described herein for the first time are crystals and three dimensional structural information from a nuclear receptor's ligand binding domain (LBD) with a ligand bound. The structure of the TR LBD complexed with 3,5,3'-triiodothyronine (T_3), 3,5-dibromo-3'-isopropylthyronine ($IpBr_2$), 3,5- dimethyl-3'-isopropylthyronine (Dimit), and 3,5,3'-triiodothyroacetic acid (Triac), 3,5-dimethyl-4-(4'-hydroxy-3'isopropylbenzyl)-phenoxy acetic acid (GC1) are exemplified. Such crystals offer superior resolution at the atomic level and the ability to visualize the coordination of nuclear receptor ligands by amino acids that comprise the LBD. The present invention also provides computational methods for designing nuclear receptor synthetic ligands using such crystal and three dimensional structural information to generate synthetic ligands that modulate the conformational changes of a nuclear receptor's LBD. Such synthetic ligands can be designed using the computational methods described herein and shown, in part, in **FIG. 1**. These computational methods are particularly useful in designing an antagonist or partial agonist to a nuclear receptor, wherein the antagonist or partial agonist has an extended moiety that prevents any one of a number of ligand-induced molecular events that alter the receptor's influence on the regulation of gene expression, such as preventing the normal coordination of the activation domain observed for a naturally occurring ligand or other ligands that mimic the naturally occurring ligand, such as an agonist. As described herein, synthetic ligands of nuclear receptors will be useful in modulating nuclear receptor activity in a variety of medical conditions.

Of particular interest is use of such ligands in a method of modulating TR activity in a mammal by administering to a mammal in need thereof a sufficient amount of a compound of Formula I,



where the compound fits spatially and preferentially into a TR LBD. By "fits spacially" is intended that the three-dimensional structure of a compound is accommodated geometrically by a cavity or pocket of a TR LBD. By "TR LBD" is intended a structural segment or segments of thyroid hormone receptor polypeptide chain folded in such a way so as to give the proper geometry and amino acid residue configuration for ligand binding. This is the physical arrangement of protein atoms in three-dimensional space forming a ligand binding pocket or cavity. By "fits spacially and preferentially" is intended that a compound possesses a three-dimensional structure and conformation for selectively interacting with a TR LBD. Compounds of interest also include derivatives of Formula I. By "derivatives of Formula I" is intended compounds that comprise at least a single phenyl scaffold (ϕ-X or X-ϕ) of the biphenyl scaffold (ϕ-X-ϕ) of Formula I which comprise the corresponding substituents of Formula I desccribed herein. Compounds that are interatively designed using structural information gleaned from these compounds and which modulate nuclear hormone receptor activity also are of interest. Preferred compounds of Formula I and its derivatives that fit spacially and preferentially into a TR LBD comprise the following substituents:

(i) an R1-substituent comprising an anionic group that interacts with a side chain nitrogen atom of an arginine corresponding to a residue from the group Arg228, Arg262, and Arg266 of human TR-α, and Arg282, Arg316 and Arg320 of human TR-β, where the anionic group is about 1.7-4.0Å from the nitrogen atom;

(ii) an R2-substituent comprising a hydrophobic or hydrophilic group that fits spacially into the TR LBD;

(iii) an R3-substituent comprising a hydrophobic or hydrophilic group that interacts with a side chain atom of a serine, alanine and/or isoleucine corresponding to a residue from the group Ser260, Ala263 and Ile299 of human TR-α, and Ser314, Ala317 and Ile352 of human TR-β, where the hydrophobic or hydrophilic group is about 1.7-4.0Å from the side chain atom;

(iv) an R5-substituent comprising a hydrophobic or hydrophilic group that interacts with a side chain atom of a phenylalanine and/or isoleucine corresponding to a residue from the group Phe218, Ile221 and Ile222 of human TR-α, and Phe272, Ile275 and Ile276 of human TR-β, where the hydrophobic or hydrophilic group is about 1.7-4.0Å from the side chain atom;

(v) an R6-substitutent comprising a hydrophobic or hydrophilic group that fits spacially into the TR LBD;

(vi) an X-substituent comprising a hydrophobic or hydrophilic group that interacts with a side chain atom of a leucine corresponding to a residue from the group Leu276 and Leu292 of human TR-α, and Leu 330 and Leu346 of human TR-β, where the hydrophobic or hydrophilic group is about 1.7-4.0Å from the side chain atom;

(vii) an R2'-substituent comprising a hydrophobic or hydrophilic group that fits spacially into the TR LBD;

(viii) an R3'-substituent comprising a hydrophobic group that interacts with a side chain atom of a phenylalanine, glycine and/or methionine corresponding to a residue from the group Phe215, Gly290, and Met388 of human TR-α, and Phe269, Gly344, Met442 of human TR-β, where the hydrophobic group is about 1.7-4.0Å from the side chain atom;

(ix) an R4'-substituent comprising an hydrogen bond donor or acceptor group that interacts with a side chain carbon or nitrogen atom of a histidine corresponding to residue His381 of human TR-α, and His435 of human TR-β, where the hydrogen bond donor or acceptor group is about 1.7-4.0Å from the side chain atom;

(x) an R5'-substituent comprising a hydrophobic or hydrophilic group that fits spacially into the TR LBD;

(xi) and R6'-substituent comprising a hydrophobic or hydrophilic group that fits spacially into the TR LBD; and

where the compound is other than thyronine (T3), triiodothyronine (T4) or other thyronine-like compounds previously known and used in a TR treatment method, such as those referenced in Appendix I.

Examples of such substituents include the following:

where R_1 is

$-O-CH_2CO_2H$, $-NHCH_2CO_2H$, $-CO_2H$, $-CH_2CO_2H$, $-CH_2CH_2CO_2H$, $-CH_2CH_2CH_2CO_2H$, $-CH_2CH(NH_2)CO_2H$, $-CH_2CH[NHCOCH\phi_2]CO_2H$, $-CH_2CH[NHCO(CH_2)_{15}CH_3]CO_2H$, $-CH_2CH[NH\text{-}FMOC]CO_2H$, $-CH_2CH[NH\text{-}tBOC]CO_2H$, or a carboxylate connected to the ring with a 0 to 3 carbon linker,

$-PO_3H_2$, $-CH_2PO_3H_2$, $-CH_2CH_2PO_3H_2$, $-CH_2CHNH_2PO_3H_2$, $-CH_2CH[NHCOCH\phi_2]PO_3H_2$, $-CH_2CH[NHCO(CH_2)_{15}CH_3]PO_3H_2$, $-CH_2CH[NH\text{-}FMOC]PO_3H_2$, $-CH_2$ $CH[NH\text{-}tBOC]PO_3H_2$, or a phosphate or phosphonate connected to the ring with a 0 to 3 carbon linker,

$-SO_3H$, $-CH_2SO_3H$, $-CH_2CH_2SO_3H$, $-CH_2CHNH_2SO_3H$, $-CH_2CH[NHCOCH\phi_2]SO_3H$, $-CH_2CH[NHCO(CH_2)_{15}CH_3]SO_3H$, $-CH_2CH[NH\text{-}FMOC]SO_3H$, $-CH_2$ CH[NH-tBOC]SO3H, or a sulfate or sulfite connected to the ring with a 0 to 3 carbon linker, or acts as the functional equivalent of $CH_2CH(NH_2)CO_2H$ of T3 in the molecular recognition domain when bound to a TR, wherein R_1 can be optionally substituted with an amine,

where R_2 is

H, halogen, CF_3, OH, NH_2, SH, CH_3, -Et, or acts as the functional equivalent of H in the molecular recognition domain when bound to a TR,

where R_3 is

-H, halogen, $-CF_3$, -OH, $-NH_2$, $-N_3$, -SH, $-CH_3$, -Et, or acts as the functional equivalent of I in the molecular recognition domain when bound to a TR,

where R_5 is

-H, halogen, $-CF_3$, -OH, $-NH_2$, $-N_3$, -SH, $-CH_3$, -Et, or acts as the functional equivalent of I in the molecular recognition domain when bound to a TR, and R_3 can be identical to R_5,

where R_6 is

-H, halogen, $-CF_3$, -OH, $-NH_2$, -SH, $-CH_3$, or acts as the functional equivalent of H in the molecular recognition domain when bound to a TR, and R_2 can be identical to R_6,

where R_2' is

-H, halogen, $-CF_3$, -OH, $-NH_2$, $-N_3$, -SH, $-CH_3$, -Et, or acts as the functional equivalent of H in the molecular recognition domain when bound to a TR,

where R_3' is any hydrophobic group, including

halogen, $-CF_3$, -SH, alkyl, aryl, 5- or 6-membered heterocyclie, cyano, or acts as the functional equivalent of I in the molecular recognition domain when bound to a TR,

where R_4' is

-H, halogen, $-CF_3$, -OH, $-NH_2$, NH_3, $-N(CH_3)_3$, carboxylate, phosphonate, phosphate or sulfate, -SH, $-CH_3$, -Et, or akyl, aryl or 5- or 6-membered heterocyclic aromatic attached through urea or carbamate linkages to O or N or S at the R_4' position, or acts as the functional equivalent of OH in the molecular recognition domain when bound to a TR,

where R_5' is

-H, -OH, $-NH_2$, $-N(CH_3)_2$ -SH $-NH_3$, $-N(CH_3)_3$, carboxylate, phosphonate, phosphate, sulfate, branched or straight chain alkyl having 1 to 9 carbons, substituted or unsubstituted aryl, wherein said substituted aryl is substituted with halogen or 1 to 5 carbon alkyl and wherein said aryl is optionally connected to the ring by a $-CH_2-$, aromatic heterocycle having 5 to 6 atoms, wherein said heterocycle may be substituted with one or more groups selected from -OH, $-NH_2$, -SH, $-NH_3$, $-N(CH_3)_3$, carboxylate, phosphonate, phosphate or sulfate, heteroalkyl, arylalkyl, heteroaryl alkyl, polyaromatic, or polyheteroaromatic, wherein said R_5' may be substituted with polar or charged groups,

where R_6' is

-H, halogen, -CF_3, -OH, -NH_2, -SH, -CH_3, -Et, or acts as the functional equivalent of H in the molecular recognition domain when bound to a TR,

where X is

O, S, SO_2, NH, NR_7, CH_2, CHR_7, CR_7R_7, wherein R_7 is alkyl, aryl or 5- or 6-membered heterocyclic aromatic,

and where the TR LBD ligand has an apparent Kd for binding TR LBD of 1 μM or less.

Of particular interest are the class of compounds according to Formula I having the following substituents: where R_1 is carboxylate, phosphonate, phosphate or sulfite and is connected to the ring with a 0 to 3 carbon linker, R_2 is H, R_3 is -I, -Br, or -CH_3, R_5 is -I, -Br, or -CH_3, R_6 is H, R_2' is H, R_3' is -I, -Br, -CH_3, -iPr, -phenyl, benzyl, or 5- or 6-membered ring heterocycles, R_4' is -OH, -NH_2, and -SH, R_5' is -H, -OH, -NH_2, -$N(CH_3)_2$ - SH -NH_3, -$N(CH_3)_3$, carboxylate, phosphonate, phosphate, sulfate, branched or straight chain alkyl having 1 to 9 carbons, substituted or unsubstituted aryl, wherein said substituted aryl is substituted with halogen or 1 to 5 carbon alkyl and wherein said aryl is optionally connected to the ring by a -CH_2-, aromatic heterocycle having 5 to 6 atoms, wherein said heterocycle may be substituted with one or more groups selected from -OH, -NH_2, -SH, -NH_3, -$N(CH_3)_3$, carboxylate, phosphonate, phosphate or sulfate, heteroalkyl, arylalkyl, heteroaryl alkyl, polyaromatic, or polyheteroaromatic, wherein said R_5' may be substituted with polar or charged groups, and R_6' is H.

The present invention also includes a method for identifying a compound capable of selectively modulating the activity of a TR isoform. By "modulating" is intended increasing or decreasing activity of a TR. By "TR isoform" is intended TR proteins encoded by subtype and variant TR genes. This includes TR-α and TR-β isoforms encoded by different genes (e.g., *thr*a and *thr*b) and variants of the same genes (e.g., *thr*b1 and *thr*b2). The method comprises the steps of modeling test compounds that fit spacially and preferentially into a TR LBD isoform of interest using an atomic structural model of a TR LBD isoform bound to a test compound, screening the test compounds in a biological assay for TR isoform activity characterized by binding of a test compound to a TR LBD isoform, and identifying a test compound that selectively modulates the activity of a TR isoform. By "modeling" is intended quantitative and qualitative analysis of receptor-ligand structure/function based on three-dimensional structural information and receptor-ligand interaction models. This

includes conventional numeric-based molecular dynamic and energy minimization models, interactive computer graphic models, modified molecular mechanics models, distance geometry and other structure-based constraint models. Modeling is preferably performed using a computer and may be further optimized using known methods.

For selectively modulating activity of a TR isoform, such as TR-α or TR-β, a sufficient amount of a compound that fits spatially and preferentially into TR LBD isoform is provided *in vitro* or *in vivo* to achieve the desired end result. TR-α isoform selectivity can be accomplished with a compound comprising an anionic group that interacts with an oxygen or carbon of a serine residue corresponding to Ser277 of human TR-α, where the anionic group is about 1.7-4.0Å from the side chain atom. TR-β isoform selectivity can be accomplished with a compound comprising an anionic group that interacts with the side chain nitrogen of an asparagine corresponding to Asn331 of human TR-β, where the anionic group is about 1.7-4.0Å from the side chain nitrogen atom.

The present invention further includes a method for identifying a TR agonist or antagonist ligand by providing the atomic coordinates of a TR LBD to a computerized modeling system, modeling ligands which fit spacially into the TR LBD, and identifying in a biological assay for TR activity a ligand which increases or decreases the activity of the TR.

The invention also involves a method for increasing receptor selectivity of a compound of Formula I or derivatives thereof for a TR-type receptor versus other nuclear receptors by selecting a compound that interacts with conformationally constrained residues of a TR LBD that are conserved among TR isoforms. "Conformationally constrained" is intended to refer to the three-dimensional structure of a chemical or moiety thereof having certain rotations about its bonds fixed by various local geometric and physical-chemical constraints. In designing and selecting compounds having increased specificity for TRs compared to other nuclear receptors, the following methods of the invention can be used. One method involves comparing atomic models of a first TR LBD isoform bound to a compound with a second TR LBD isoform bound to the same compound, identifying atoms of the TR LBD and compounds which interact, and designing or selecting a compound that interacts with TR LBD residues comprising a conformationally constrained structural feature that is conserved between the TR LBD isoforms. Another method relates to comparing a first TR LBD complexed with a first compound to a second TR LBD complexed with a

second compound having one or more different substituents compared to the first compound, identifying atoms of the TR LBD and compounds which interact, and designing or selecting compounds that interact with TR LBD residues comprising a conformationally constrained structural feature that is conserved between the TR LBD isoforms. The methods also facilitate identification of structural and conformationally constrained interactions that are conserved between compounds that bind to a TR LBD. The methods are exemplified by comparing atomic models of a first TR LBD isoform complexed with a first compound of Formula I to a second TR LBD isoform complexed with the first compound, or a second compound of Formula I having different substituents than the first compound. For example, a TR-α LBD bound to a natural hormone such as T3 is compared to a TR-β LBD bound to an organic thyronine-like compound such as GC-1. Conserved contacts are identified which are made between atoms of the different compounds and atoms of the TR LBDs, and the fiducial and adjustable components identified. Compounds selective for TR are identified in a biological assay for TR activity that assays for selective binding to a TR and/or TR LBD compared to other nuclear receptors. Conventional assays for TR and other nuclear receptors may be conducted in parallel or serially, including those assays described herein. Automatable methods are preferred. The methods facilitate design and selection of compounds comprising cyclic carbon and substituent atoms that interact with a constrained side chain and/or main chain atom of a TR LBD residue.

In another aspect of the invention, the methods described herein are useful for selecting peptides, peptidomimetics or synthetic molecules that modulate TR activity. Methods of the invention also find use in characterizing structure/function relationships of natural and synthetic TR-ligands. Molecules of particular interest are new thyronine-like compounds other than T3, T4 and other thyronine-like compounds previously known and used for treating TR-related disorders. New compounds of the invention include those which bind to a TR LBD isoform with greater affinity than T3 or T4 and those which exhibit isoform-specific binding affinity.

APPLICABILITY TO NUCLEAR RECEPTORS

The present invention, particularly the computational methods, can be used to design drugs for a variety of nuclear receptors, such as receptors for glucocorticoids (GRs),

androgens (ARs), mineralocorticoids (MRs), progestins (PRs), estrogens (ERs), thyroid hormones (TRs), vitamin D (VDRs), retinoid (RARs and RXRs), icosanoid (IRs), and peroxisomes (XPARS and peroxisomal proliferators (PPAP)). The present invention can also be applied to the "orphan receptors," as they are structurally homologous in terms of modular domains and primary structure to classic nuclear receptors, such as steroid and thyroid receptors. The amino acid homologies of orphan receptors with other nuclear receptors ranges from very low (<15%) to in the range of 35% when compared to rat RARα and human TR-β receptors, for example. In addition, as is revealed by the X-ray crystallographic structure of the TR and structural analysis disclosed herein, the overall folding of liganded superfamily members is likely to be similar. Although ligands have not been identified with orphan receptors, once such ligands are identified one skilled in the art will be able to apply the present invention to the design and use of such ligands, as their overall structural modular motif will be similar to other nuclear receptors described herein.

Modular Functional Domains Of Nuclear receptors

The present invention will usually be applicable to all nuclear receptors, as discussed herein, in part, to the patterns of nuclear receptor activation, structure and modulation that have emerged as a consequence of determining the three dimensional structures of nuclear receptors with different ligands bound, notably the three dimensional structures or crystallized protein structure of the ligand binding domains for TR-α and TR-β. Proteins of the nuclear receptor superfamily display substantial regions of amino acid homology, as described herein and known in the art see **FIG. 2**. Members of this family display an overall structural motif of three modular domains (which is similar to the TR three modular domain motif):

1) a variable amino-terminal domain;

2) a highly conserved DNA-binding domain (DBD); and

3) a less conserved carboxyl-terminal LBD.

The modularity of this superfamily permits different domains of each protein to separately accomplish different functions, although the domains can influence each other. The separate function of a domain is usually preserved when a particular domain is isolated from the remainder of the protein. Using conventional protein chemistry techniques a modular domain

can sometimes be separated from the parent protein. Using conventional molecular biology techniques each domain can usually be separately expressed with its original function intact or chimerics of two different nuclear receptors can be constructed, wherein the chimerics retain the properties of the individual functional domains of the respective nuclear receptors from which the chimerics were generated.

FIG. 2 provides a schematic representation of family member structures, indicating regions of homology within family members and functions of the various domains.

Amino Terminal Domain

The amino terminal domain is the least conserved of the three domains and varies markedly in size among nuclear receptor superfamily members. For example, this domain contains 24 amino acids in the VDR and 603 amino acids in the MR. This domain is involved in transcriptional activation and in some cases its uniqueness may dictate selective receptor-DNA binding and activation of target genes by specific receptor isoforms. This domain can display synergistic and antagonistic interactions with the domains of the LBD. For example, studies with mutated and/or deleted receptors show positive cooperativity of the amino and carboxy terminal domains. In some cases, deletion of either of these domains will abolish the receptor's transcriptional activation functions.

DNA-Binding Domain

The DBD is the most conserved structure in the nuclear receptor superfamily. It usually contains about 70 amino acids that fold into two zinc finger motifs, wherein a zinc ion coordinates four cysteines. DBDs contain two perpendicularly oriented α-helixes that extend from the base of the first and second zinc fingers. The two zinc fingers function in concert along with non-zinc finger residues to direct nuclear receptors to specific target sites on DNA and to align receptor homodimer or heterodimer interfaces. Various amino acids in DBD influence spacing between two half-sites (usually comprised of six nucleotides) for receptor dimer binding. For example, GR subfamily and ER homodimers bind to half-sites spaced by three nucleotides and oriented as palindromes. The optimal spacings facilitate cooperative interactions between DBDs, and D box residues are part of the dimerization

interface. Other regions of the DBD facilitate DNA-protein and protein-protein interactions required for RXR homodimerization and heterodimerization on direct repeat elements.

The LBD may influence the DNA binding of the DBD, and the influence can also be regulated by ligand binding. For example, TR ligand binding influences the degree to which a TR binds to DNA as a monomer or dimer. Such dimerization also depends on the spacing and orientation of the DNA half sites. The receptors also can interact with other proteins and function to regulate gene expression.

The nuclear receptor superfamily has been subdivided into two subfamilies: 1) GR (GR, AR, MR and PR) and 2) TR (TR, VDR, RAR, RXR, and most orphan receptors) on the basis of DBD structures, interactions with heat shock proteins (hsp), and ability to form heterodimers. GR subgroup members are tightly bound by hsp in the absence of ligand, dimerize following ligand binding and dissociation of hsp, and show homology in the DNA half sites to which they bind. These half sites also tend to be arranged as palindromes. TR subgroup members tend to be bound to DNA or other chromatin molecules when unliganded, can bind to DNA as monomers and dimers, but tend to form heterodimers, and bind DNA elements with a variety of orientations and spacings of the half sites, and also show homology with respect to the nucleotide sequences of the half sites. By this classification, ER does not belong to either subfamily, since it resembles the GR subfamily in hsp interactions, and the TR subfamily in nuclear localization and DNA-binding properties.

Ligand Binding Domain

The LBD is the second most highly conserved domain in these receptors. Whereas integrity of several different LBD sub-domains is important for ligand binding, truncated molecules containing only the LBD retain normal ligand-binding activity. This domain also participates in other functions, including dimerization, nuclear translocation and transcriptional activation, as described herein. Importantly, this domain binds the ligand and undergoes ligand-induced conformational changes as detailed herein.

Most members of the superfamily, including orphan receptors, possess at least two transcription activation subdomains, one of which is constitutive and resides in the amino terminal domain (AF-1), and the other of which (AF-2 (also referenced as TAU 4)) resides in the ligand-binding domain whose activity is regulated by binding of an agonist ligand.

The function of AF-2 requires an activation domain (also called transactivation domain) that is highly conserved among the receptor superfamily (approximately amino acids 1005 to 1022). Most LBDs contain an activation domain. Some mutations in this domain abolish AF-2 function, but leave ligand binding and other functions unaffected. Ligand binding allows the activation domain to serve as an interaction site for essential co-activator proteins that function to stimulate (or in some cases, inhibit) transcription.

For example, Shibata, H., *et al.* (*Recent Progress in Hormone Res.* 52:141-164 (1997)) has reviewed the role of co-activators and co-repressors in steroid/thyroid hormone receptor systems. Steroid receptor co-activator-one (SRC-1) appears to be a general co-activator for all AF-2 domain containing receptors tested. SRC-1 enhances transactivation of steroid hormone-dependent target genes. Other putative co-activators have been reported, including the SRC-1 related proteins, TIF-2 and GRIP-1, and other putative unrelated co-activators such as ARA-70, Trip 1, RIP-140, and TIF-1. In addition another co-activator CREB-binding protein (CBP) has been shown to enhance receptor-dependent target gene transcription. CBP and SRC-1 interact and synergistically enhance trancriptional activation by the ER and PR. A ternary complex of CBP, SRC-1, and liganded receptors-may form to increase the rate of hormone-responsive gene transcription. Co-repressors, such as SMRT and N-CoR, for TR and RAR, have been identified that also contribute to the silencing function of unliganded TR. The unliganded TR and RAR have been shown to inhibit basal promoter activity; this silencing of target gene transcription by unliganded receptors is mediated by these co-repressors. The collective data suggests that upon binding of agonist, the receptor changes its conformation in the ligand-binding domain that enables recruitment of co-activators, which allows the receptor to interact with the basal transcriptional machinery more efficiently and to activate transcription. In contrast, binding of antagonists induces a different conformational change in the receptor. Although some antagonist-bound receptors can dimerize and bind to their cognate DNA elements, they fail to dislodge the associated co-repressors, which results in a nonproductive interaction with the basal transcriptional machinery. Similarly, the TR and RAR associate with co-repressors in the absence of ligand, thereby resulting in a negative interaction with the transcriptional machinery that silences target gene expression. In the case of mixed agonist/antagonists, such as 4-hydroxytamoxifen, activation of gene transcription may depend on the relative ratio

of co-activators and co-repressors in the cell or cell-specific factors that determine the relative agonistic or antagonistic potential of different compounds. These co-activators and co-repressors appear to act as an accelerator and/or a brake that modulates transcriptional regulation of hormone-responsive target gene expression.

The carboxy-terminal activation subdomain, as described herein is in close three dimensional proximity in the LBD to the ligand, so as to allow for ligands bound to the LBD to coordinate (or interact) with amino acid(s) in the activation subdomain. As described herein, the LBD of a nuclear receptor can be expressed, crystallized, its three dimensional structure determined with a ligand bound (either using crystal data from the same receptor or a different receptor or a combination thereof), and computational methods used to design ligands to its LBD, including ligands that contain an extension moiety that coordinates the activation domain of the nuclear receptor.

Once a computationally designed ligand (CDL) is synthesized as described herein and known in the art, it can be tested using assays to establish its activity as an agonist, partial agonist or antagonist, and affinity, as described herein. After such testing, the CDLs can be further refined by generating LBD crystals with a CDL bound to the LBD. The structure of the CDL can then be further refined using the chemical modification methods described herein for three dimensional models to improve the activity or affinity of the CDL and make second generation CDLs with improved properties, such as that of a super agonist or antagonist described herein. Agonist and antagonist ligands also can be selected that modulate nuclear receptor responsive gene transcription through altering the interaction of co-activators and co-repressors with their cognate nuclear hormone receptor. For example, CDL agonists can be selected that block or dissociate the co-repressor from interaction with the receptor, and/or which promote binding or association of the co-activator. CDL antagonists can be selected that block co-activator interaction and/or promote co-repressor interaction with the target receptor. Selection can be done in binding assays that screen for CDLs having the desired agonist or antagonist properties. Suitable assays for such screening are described herein and in Shibata, H., *et al.* (*Recent Prog. Horm. Res. 52*:141-164 (1997)); Tagami, T., *et al.* (*Mol. Cell Biol. 17*(5):2642-2648 (1997)); Zhu, XG., *et al.* (*J. Biol. Chem. 272*(14):9048-9054 (1997)); Lin, B.C., *et al.* (*Mol. Cell Biol. 17*(10):6131-6138 (1997)); Kakizawa, T., *et al.* (*J. Biol. Chem. 272*(38):23799-23804 (1997)); and Chang, K.

H., *et al.* (*Proc. Natl. Acad. Sci. USA* 94(17):9040-9045 (1997)), which references are incorporated herein in their entirety by reference.

NUCLEAR RECEPTOR ISOFORMS

The present invention also is applicable to generating new synthetic ligands to distinguish nuclear receptor isoforms. As described herein, CDLs can be generated that distinguish between binding isoforms, thereby allowing the generation of either tissue specific or function specific synthetic ligands. For instance, GR subfamily members have usually one receptor encoded by a single gene, although are exceptions. For example, there are two PR isoforms, A and B, translated from the same mRNA by alternate initiation from different AUG codons. There are two GR forms, one of which does not bind ligand. This method is especially applicable to the TR subfamily which usually has several receptors that are encoded by at least two (TR: α, β) or three (RAR, RXR, and PPAR: α, β, γ) genes or have alternate RNA splicing and such an example for TR is described herein.

NUCLEAR RECEPTOR CRYSTALS

The invention provides for crystals made from nuclear receptor ligand binding domains with the ligand bound to the receptor. As exemplified in the Examples, TRs are crystallized with a ligand bound to it. Crystals are made from purified nuclear receptor LBDs that are usually expressed by a cell culture, such as *E. coli*. Preferably, different crystals (co-crystals) for the same nuclear receptor are separately made using different ligands, such as a naturally occurring ligand and at least one bromo- or iodo- substituted synthetic ligand that acts as an analog or antagonist of the naturally occurring ligand. Such bromo- and iodo- substitutions act as heavy atom substitutions in nuclear receptor ligands and crystals of nuclear receptor proteins. This method has the advantage for phasing of the crystal in that it bypasses the need for obtaining traditional heavy metal derivatives. After the three dimensional structure is determined for the nuclear receptor LBD with its ligand bound, the three dimensional structure can be used in computational methods to design a synthetic ligand for the nuclear receptor and further activity structure relationships can be determined through routine testing using the assays described herein and known in the art.

Expression and Purification of other Nuclear Receptor LBD Structures

High level expression of nuclear receptor LBDs can be obtained by the techniques described herein as well as others described in the literature. High level expression in E. coli of ligand binding domains of TR and other nuclear receptors, including members of the steroid/thyroid receptor superfamily, such as the receptors ER, AR, MR, PR, RAR, RXR and VDR can also be achieved. Yeast and other eukaryotic expression systems can be used with nuclear receptors that bind heat shock proteins as these nuclear receptors are generally more difficult to express in bacteria, with the exception of ER, which can be expressed in bacteria. Representative nuclear receptors or their ligand binding domains have been cloned and sequenced: human RAR-α, human RAR-γ, human RXR-α, human RXR-β, human PPAR-α, human PPAR-β, human PPAR-γ, human VDR, human ER (as described in Seielstad *et al., Molecular Endocrinology, vol 9*:647-658 (1995), incorporated herein by reference), human GR, human PR, human MR, and human AR. The ligand binding domain of each of these nuclear receptors has been identified and is shown in **FIG. 3**. Using the information in **FIG. 3** in conjunction with the methods described herein and known in the art, one of ordinary skill in the art could express and purify LBDs of any of the nuclear receptors, including those illustrated in **FIG. 3**, bind it to an appropriate ligand, and crystallize the nuclear receptor's LBD with a bound ligand.

FIG. 3 is an alignment of several members of the steroid/thyroid hormone receptor superfamily that indicates the amino acids to be included in a suitable expression vector.

Extracts of expressing cells are a suitable source of receptor for purification and preparation of crystals of the chosen receptor. To obtain such expression, a vector is constructed in a manner similar to that employed for expression of the rat TR alpha (Apriletti *et al. Protein Expression and Purification, 6*:363-370 (1995), herein incorporated by reference). The nucleotides encoding the amino acids encompassing the ligand binding domain of the receptor to be expressed, for example the estrogen receptor ligand binding domain (hER-LBD) (corresponding to R at position 725 to L at position 1025 as standardly aligned as shown in the **FIG. 3**), are inserted into an expression vector such as the one employed by Apriletti et al (1995). For the purposes of obtaining material that will yield good crystals it is preferable to include at least the amino acids corresponding to human TR-β positions 725 to 1025. Stretches of adjacent amino acid sequences may be included if

more structural information is desired. Thus, an expression vector for the human estrogen receptor can be made by inserting nucleotides encoding amino acids from position 700 to the c-terminus at position 1071. Such a vector gives high yield of receptor in E. coli that can bind hormone (Seielstad *et al. Molecular Endocrinology 9*:647-658 (1995)). However, the c-terminal region beyond position 1025 is subject to variable proteolysis and can advantageously be excluded from the construct, this technique of avoiding variable proteolysis can also be applied to other nuclear receptors.

TR-α And TR-β As Examples of Nuclear receptor LBD Structure and Function

TR Expression, Purification And Crystallization

As an example of nuclear receptor structure of the ligand binding domain the α- and β- isoforms of TR are crystallized from proteins expressed from expression constructs, preferably constructs that can be expressed in E. coli. Other expression systems, such as yeast or other eukaryotic expression systems can be used. For the TR, the LBD can be expressed without any portion of the DBD or amino-terminal domain. Portions of the DBD or amino-terminus can be included if further structural information with amino acids adjacent the LBD is desired. Generally, for the TR the LBD used for crystals will be less than 300 amino acids in length. Preferably, the TR LBD will be at least 150 amino acids in length, more preferably at least 200 amino acids in length, and most preferably at least 250 amino acids in length. For example the LBD used for crystallization can comprise amino acids spanning from Met 122 to Val 410 of the rat TR-α, Glu 202 to Asp 461 of the human TR-β.

Typically TR LBDs are purified to homogeneity for crystallization. Purity of TR LBDs is measured with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), mass spectrometry (MS) and hydrophobic high performance liquid chromatography (HPLC). The purified TR for crystallization should be at least 97.5 % pure or 97.5%, preferably at least 99.0% pure or 99.0% pure, more preferably at least 99.5% pure or 99.5% pure.

Initially purification of the unliganded receptor can be obtained by conventional techniques, such as hydrophobic interaction chromatography (HPLC), ion exchange chromatography (HPLC), and heparin affinity chromatography.

To achieve higher purification for improved crystals of nuclear receptors, especially the TR subfamily and TR, it will be desirable to ligand shift purify the nuclear receptor using a column that separates the receptor according to charge, such as an ion exchange or hydrophobic interaction column, and then bind the eluted receptor with a ligand, especially an agonist. The ligand induces a change in the receptor's surface charge such that when re-chromatographed on the same column, the receptor then elutes at the position of the liganded receptor are removed by the original column run with the unliganded receptor. Usually saturating concentrations of ligand are used in the column and the protein can be preincubated with the ligand prior to passing it over the column. The structural studies detailed herein indicate the general applicability of this technique for obtaining super-pure nuclear receptor LBDs for crystallization.

More recently developed methods involve engineering a "tag" such as with histidine placed on the end of the protein, such as on the amino terminus, and then using a nickle chelation column for purification, Janknecht R., *Proc. Natl. Acad. Sci. USA, 88*:8972-8976-(1991) incorporated by reference.

To determine the three dimensional structure of a TR LBD, or a LBD from another member of the nuclear receptor superfamily, it is desirable to co-crystalize the LBD with a corresponding LBD ligand. In the case of TR LBD, it is preferable to separately co-crystalize it with ligands such as T3, IpBr and Dimit that differ in the heavy atoms which they contain. Other TR ligands such as those encompassed by Formula 1 described herein and known in the prior art, can also be used for the generation of co-crystals of TR LBD and TR ligands. Of the compounds encompassed by Formula 1 it is generally desirable to use at least one ligand that has at least one bromo- or iodo- substitution at the R_3, R_5, R_3' or R_5' position, preferably such compounds will be have at least two such substitutions and more preferably at least 3 such substitutions. As described herein, such substitutions are advantageously used as heavy atoms to help solve the phase problem for the three dimensional structure of the TR LBD and can be used as a generalized method of phasing using a halogen (e.g. I or Br) substituted ligand, especially for nuclear receptors.

Typically purified LBD, such as TR LBD, is equilibrated at a saturating concentration of ligand at a temperature that preserves the integrity of the protein. Ligand equilibration

can be established between 2 and 37°C, although the receptor tends to be more stable in the 2-20°C range.

Preferably crystals are made with the hanging drop methods detailed herein. Regulated temperature control is desirable to improve crystal stability and quality. Temperatures between 4 and 25°C are generally used and it is often preferable to test crystallization over a range of temperatures. In the case of TR it is preferable to use crystallization temperatures from 18 to 25°C, more preferably 20 to 23°C, and most preferably 22°C.

Complexes of the TR-α LBD with a variety of agonists, including T_3, $IpBr_2$, Dimit, and Triac, are prepared with by methods described herein. For example, cocrystals of the rTR-α LBD, with ligand prebound, are prepared by vapor diffusion at ambient temperature from 15% 2-methyl-2,4-pentanediol (MPD). The crystals are radiation sensitive, and require freezing to measure complete diffraction data. On a rotating anode X-ray source, the crystals diffract to ~3Å; synchrotron radiation extends the resolution limit significantly, to as high as 2.0Å for T_3 cocrystals. The composition of the thyroid hormone, combined with the ability to prepare and cocrystallize the receptor complexed with a variety of analogs, permitted the unusual phasing strategy. This phasing strategy can be applied to the ligands of the nuclear receptors described therein by generating I and Br substitutions of such ligands. In this strategy, cocrystals of the TR LBD containing four hormone analogs that differ at the 3,5, and 3' positions (T_3, $IpBr_2$, Dimit, and Triac) provided isomorphous derivatives. For this set of analogs, the halogen substituents (2Br and 3I atoms) function as heavy atoms, while the Dimit cocrystal (3 alkyl groups) acts as the parent. The initial 2.5Å multiple isomorphous replacement/anomalous scattering/density modified electron density map allowed the LBD to be traced from skeletons created in the molecular graphics program O5 (Jones, T.A. *et al.*, *ACTA Cryst, 47*:110-119 (1991), incorporated by reference herein). A model of the LBD was built in four fragments, Arg157-Gly184, Trp186-Gly197, Ser199-Pro205, and Val210-Phe405, and refined in XPLOR using positional refinement and simulated annealing protocols. Missing residues were built with the aid of difference density. The final model was refined to R_{cryst} = 21.8% and R_{free} = 24.4% for data from 15.0 to 2.2Å, see **Table 6**. The human TR-β LBD model was resolved by molecular replacement of the TR-α LBD coordinates. The structure is based on E202 to D461 with a

his-tag at the N-terminus. The final model was refined to R_{cryst} = 25.3% and R_{free} = 28.9% for data from 30.0 to 2.4Å+, see Table 7.

This phasing strategy can be applied to the ligands of the nuclear receptors described herein by generating I and Br substitutions of such ligands.

THREE DIMENSIONAL STRUCTURE OF TR LBD

Architecture of TR LBD

As an example of the three dimensional structure of a nuclear receptor, the folding of the TR-α_1 LBD is shown in **FIG. 4**. The TR-α LBD consists of a single structural domain packed in three layers, composed of twelve α-helices, H1-12, and four short β-strands, S1-4, forming a mixed β-sheet. The buried hormone and three antiparallel α-helices, H5-6, H9, and H10, form the central layer of the domain, as shown in **FIG. 4**. H1, H2, H3 and S1 form one face of the LBD, with the opposite face formed by H7, H8, H11, and H12. The first 35 amino acids of the N-terminus (Met122-Gln156) are not visible in the electron density maps. The three dimensional structure of the heterodimeric RXR:TR DNA-binding domains bound to DNA, amino acids Met 122 - Gln151 of the TR DBD make extensive contacts with the minor groove of the DNA8. The five disordered amino acids (Arg152-Gln156), which reside between the last visible residue of the TR DBD and the first visible residue of the LBD likely represent the effective "hinge" linking the LBD and the DBD in the intact receptor.

The predominantly helical composition and the layered arrangement of secondary structure is identical to that of the unliganded hRXRα, confirming the existence of a common nuclear receptor fold between two nuclear receptors.

The TR LBD is visible beginning at Arg157, and continues in an extended coil conformation to the start of H1. A turn of α-helix, H2, covers the hormone binding cavity, immediately followed by short β-strand, S1, which forms the edge of the mixed β-sheet, parallel to S4, the outermost of the three antiparallel strands. The chain is mostly irregular until H3 begins, antiparallel to H1. H3 bends at Ile221 and Ile222, residues which contact the ligand. The chain turns almost 90° at the end of H3 to form an incomplete α-helix, H4. The first buried core helix, H5-6, follows, its axis altered by a kink near the ligand at Gly 253. The helix is composed of mostly hydrophobic sidechains interrupted by two striking

exceptions: Arg262 is solvent inaccessible and interacts with the ligand carboxylate (1-substituent), and Glu256 meets Arg329 from H9 and Arg375 from H11 in a polar invagination. H5-6 terminates in a short β-strand, S2, of the four strand mixed sheet. S3 and S4 are joined through a left-handed turn, and further linked by a salt bridge between Lys284 and Asp272. Following S4, H7 and H8 form an L, stabilized by a salt bridge between Lys268 and Asp277. The turn between H7 and H8 adopts an unusual conformation, a result of interaction with ligand and its glycine rich sequence. H9 is the second core helix. antiparallel to the neighboring H5-6. Again, two buried polar sidechains are found, Glu315 and Gln320. Glu315 forms a buried salt bridge with His358 and Arg356. The oxygen of Gln320 forms a hydrogen bond with the buried sidechain of His 175. The chain then switches back again to form H10, also antiparallel to H9. H11 extends diagonally across the full length of the molecule. Immediately after H11, the chain forms a type II turn, at approximately 90° to H11. The chain then turns again to form H 12, which packs loosely against H3 and H11 as part of the hormone or ligand binding cavity. The final five amino - acids at the C-terminus, Glu406 -Val410, are disordered. The architecture of the TR-β LBD is identical to that of the TR-α LBD, with two significant differences. An additional helix is present at the N-terminus (residues Glu202-I1e208), which is part of the DBD, and packs antiparallel to H10. Following the helix is a two residue turn (Gly209-His210) continuing into an extended coil to he start of H1, as seen in the TR-α LBD. A further difference occurs in the irregular conformation adopted between H2 and H3. In the TR-α LBD, residue Gly197-Asp211 form a loop that packs against the receptor, contacting helices H7, H8, H11, and the loop between H11 and H12. In the TR-β LBD, only the ends of the loop are ordered, with the stretch Ala253-Lys263 disordered. In addition to these residues, the residues of the His-tag at the N-terminus, and the final residue at the C-terminus, Asp461, are disordered.

TR LBD's Ligand Binding Cavity As An Example Of A Nuclear Receptor's Buried Ligand Cavity

The three dimensional structure of the TR LBD leads to the startling finding that ligand binding cavity of the LBD is solvent inaccessible when a T3 or its isostere is bound to the LBD. This surprising result leads to a new model of nuclear receptor three dimensional

structure and function, as further described herein, particularly in the sections elucidating the computational methods of ligand design and the application of such methods to designing nuclear receptor synthetic ligands that contain extended positions that prevent normal activation of the activation domain.

Dimit, the ligand bound to the receptor, is an isostere of T_3 and a thyroid hormone agonist. Therefore the binding of Dimit should reflect that of T_3, and the Dimit-bound receptor is expected to be the active conformation of TR. The ligand is buried within the receptor, providing the hydrophobic core for a subdomain of the protein, as shown in **FIG. 5 a and b**. H5-6 and H9 comprise the hydrophobic core for the rest of the receptor.

An extensive binding cavity is constructed from several structural elements. The cavity is enclosed from above by H5-6 (Met 256- Arg266), from below by H7 and H8 and the intervening loop (Leu287- Ile299), and along the sides by H2 (185-187), by the turn between S3 and S4 (Leu276-Ser277), by H3 (Phe215-Arg228), by H11 (His381-Met388) and by H12 (Phe401-Phe405). The volume of the cavity defined by these elements, calculated by GRASP (Columbia University, USA) (600 Å3), is essentially the volume of the hormone (530 Å). The change in volume can be exploited for ligand design as described herein. The remaining volume is occupied by water molecules surrounding the amino-propionic acid substituent. **FIG. 6** depicts various contacts (or interactions) between TR's LBD and the ligand.

The planes of the inner and outer (prime ring) rings of the ligand are rotated from planarity about 60° with respect to each other, adopting the 3'-distal conformation (in which the 3' substituent of the outer ring projects down and away from the inner ring). The amino-propionic acid and the outer phenolic ring assume the transoid conformation, each on opposite sides of the inner ring. The torsion angle χ_1 for the amino- propionic acid is 300°.

The amino-propionic acid substituent is packed loosely in a polar pocket formed by side chains from H2, H4 and S3. The carboxylate group forms direct hydrogen bonds with the guanidium group of Arg228 and the amino N of Ser277. In addition, Arg262, Arg266 and Asn179 interact with the carboxylate through water-mediated hydrogen bonds. The three arginine residues create a significantly positive local electrostatic potential, which may stabilize the negative charge of the carboxylate. No hydrogen bond is formed by the amino nitrogen. The interactions of the amino-propionic acid substituent are consistent with the fact

that Triac, which lacks the amino nitrogen, has a binding affinity equal to that of T_3, indicating that the amino nitrogen and longer aliphatic chain of T_3 do not contribute greatly to binding affinity.

The biphenyl ether, in contrast, is found buried within the hydrophobic core. The inner ring packs in a hydrophobic pocket formed by H3, H5-6, and S3. Pockets for the 3- and 5-methyl substituents are not completely filled, as expected since the van der waals radius of methyl substituent for Dimit is smaller than the iodine substituent provided by the thyroid hormone T_3. Such pockets are typically 25 to 100 cubic angstroms (although smaller pocket for substitutes are contemplated in the 40 to 80 cubic angstrom range) and could be filled more tightly with better fitting chemical substitutions, as described herein.

The outer ring packed tightly in a pocket formed by H3, H5-6, H7, H8, H11 and H12, and the loop between H7 and H8. The ether oxygen is found in a hydrophobic environment defined by Phe218, Leu287, Leu276, and Leu292. The absence of a hydrogen bond to the ether oxygen is consistent with its role in establishing the correct stereochemistry of the phenyl rings, as suggested by potent binding of hormone analogs with structurally similar linkages possessing reduced or negligible hydrogen bonding capability. The 3'-isopropyl substituent contacts Gly290 and 291. The presence of glycine at this position in the pocket can explain the observed relationship between activity and the size of 3'-substituents. Activity is highest for 3'-isopropyl, and decreases with added bulk. The only hydrogen bond in the hydrophobic cavity is formed between the phenolic hydroxyl and His381 Nϵ2. The conformation of His381 is stabilized by packing contacts provided by Phe405, and Met256.

The presence of a 5' substituent larger than hydrogen affects the binding affinity for hormone. The more abundant thyroid hormone, 3,5,3',5'-tetraiodo-L-thyronine (T_4), contains an iodine at this position, and binds the receptor with 2% of the affinity of T_3. The structure suggests that discrimination against T_4 is accomplished through the combination of steric conflict by Met256 and possibly the constraints imposed by the geometry of the hydrogen bond from His381 to the phenolic hydroxyl. The 5' position is a preferred location for introducing a chemical modification of C-H at the 5' of T3 or and TR agonist, as described herein, that produces an extension from the prime ring and results in the creation of an antagonist or partial agonist.

Deletion and antibody competition studies suggest the involvement of residues Pro162 to Val202 in ligand binding. The region does not directly contact hormone in the bound structure, although H2 packs against residues forming the polar pocket that interacts with the amino-propionic acid group. One role for H2, then, is to stabilize these residues in the bound state, H2, with β-strands S3 and S4, might also represent a prevalent entry point for ligand, since the amino-propionic acid of the ligand is oriented toward this region. Studies of receptor binding to T_3 affinity matrices demonstrate that only a linkage to the amino-propionic acid is tolerated, suggesting that steric hindrance present in other linkages prevent binding. Furthermore, the crystallographic temperature factors suggest the coil and β-strand region is most flexible part of the domain **FIG. 7**. Participation of this region, part of the hinge domain between the DBD and LBD, in binding hormone may provide structural means for ligand binding to influence DNA binding, since parts of the Hinge domain contact DNA.

TR LBD Transcriptional Activation Helix As An Example Of A Nuclear Receptor Activation Domain

In addition to the startling finding that the ligand binding cavity is solvent inaccessible when loaded with a ligand, the activation helix of TR LBD presents a surface to the ligand cavity for interaction between at least one amino acid and the bound ligand. The C-terminal 17 amino acids of the TR, referred to as the activation helix or AF-2 (an example of an LBD activation domain), are implicated in mediating hormone-dependent transcriptional activation. Although, mutations of key residues within the domain decrease ligand-dependent activation it was unclear until the present invention whether such mutations directly affected ligand coordination. Although some mutations of this domain have been noted to reduce or abolish ligand binding, other mutations in more distant sites of the LBD have a similar effect.

Activation domains among nuclear receptors display an analogous three dimensional relationship to the binding cavity, which is a region of the LBD that binds the molecular recognition domain of a ligand, i.e. the activation domain presents a portion of itself to the binding cavity (but necessarily the molecular recognition domain of the ligand). Many nuclear receptors are expected to have such domains, including the retinoid receptors, RAR and RXR, the glucocorticoid receptor GR, and the estrogen receptor ER. Based upon the TR's sequence, the domain is proposed to adopt an amphipathic helical structure. β-sheet or

mixed secondary structures, could be present as activation domains in less related nuclear receptors.

Within the activation domain, the highly conserved motif ΦΦXEΦΦ, where Φ represents a hydrophobic residue, is proposed to mediate interactions between the receptors and transcriptional coactivators. Several proteins have been identified which bind the TR in a hormone-dependent fashion. One of these, Trip1, is related to a putative yeast coactivator Sug1, and also interacts with both the C-terminal activation domain and a subset of the basal transcriptional machinery, suggesting a role in transactivation by the TR. Other proteins, such as RIP140, SRC1, (Onate, S.A. et. al., *Science* 270:1354-1357 (1995)) and TF-1 (see also Ledouarim, B., et. al., *EMBO J.* 14:2020-2033 (1995)), and GRIP-1 (Heery, E., *et al.*, *Nature 387*:733-736 (1997)) also interact with other nuclear receptors in a ligand dependent manner through the C-terminal domain. Binding of these proteins can be modulated using the TR ligands described herein especially those TR ligands with extensions that sterically hinder the interaction between the highly conserved motif and other proteins.

The C-terminal activation domain of the TR forms an amphipathic helix, H12, which nestles loosely against the receptor to form part of the hormone binding cavity. The helix packs with the hydrophobic residues facing inward towards the hormone binding cavity, and the charged residues, including the highly-conserved glutamate, extending into the solvent, as shown in **FIG. 8.** The activation helix of TR LBD presents Phe 401 to the ligand binding cavity and permits direct coordination with the hormone i.e. such amino acids interact with the ligand forming a van der waals contact with the plane of the outer phenyl ring. Phe 405 also interacts with His 381, perhaps stabilizing its hydrogen bonding conformation, i.e. a favorable hydrogen bond interaction. Participation of Phe 401 and Phe 405 in binding hormone explains how mutation of these residues decreases hormone binding affinity. Furthermore, the impact of these mutations on activation likely derives from a role in stabilizing the domain in the bound structure through increased hydrogen bond interaction of dipole interactions. Glu 403 extends into the solvent, emphasizing its critical role in transactivation. In its observed conformation, presented on the surface as an ordered residue, against a background of predominantly hydrophobic surface, Glu 403 is available to interact with activator proteins described herein, as shown in **FIG. 9.** The other charged residues, Glu 405 and Asp 406 are disordered, as the helix frays at Phe 405.

Two other sequences in the TR, $\tau 2$ and $\tau 3$, activate transcription when expressed as fusion proteins with a DNA-binding domain. The sequences, discovered in the TRB, correspond to TR-α residues Pro158-Ile168 in H1 ($\tau 2$), and Gly290-Leu3 19 in H8 and H9 ($\tau 3$). Unlike the C-terminal activation domain, $\tau 2$ and $\tau 3$ do not appear to represent modular structural units in the rat TR-α LBD, nor present a surface for protein-protein interactions: the critical aspartate/glutamate residues of $\tau 3$ are located on two separate helices, and do not form a single surface; the charged residues of $\tau 2$ are engaged in ion pair interactions with residues of the LBD. Thus, $\tau 2$ and $\tau 3$ may not function as activation domains in the context of the entire receptor.

Computational Methods For Designing A Nuclear Receptor LBD LIGAND

The elucidation of the three dimensional structure of a nuclear receptor ligand binding domain provides an important and useful approach for designing ligands to nuclear receptors using the computational methods described herein. By inspecting the **FIGURES** it can be determined that the nuclear receptor ligand is bound in a water inaccessible binding cavity in the LBD and that chemical moieties can be added to selected positions on the ligand. Such chemical modifications, usually extensions, can fill up the binding cavity represented in the **FIGURES** for a tighter fit (or less water) or can be used to disrupt or make contacts with amino acids not in contact with the ligand before the chemical modification was introduced or represented in a figure of the three dimensional model of the LBD. Ligands that interact with nuclear superfamily members can act as agonists, antagonists and partial agonists based on what ligand-induced conformational changes take place.

Agonists induce changes in receptors that place them in an active conformation that allows them to influence transcription, either positively or negatively. There may be several different ligand-induced changes in the receptor's conformation.

Antagonists, bind to receptors, but fail to induce conformational changes that alter the receptor's transcriptional regulatory properties or physiologically relevant conformations. Binding of an antagonist can also block the binding and therefore the actions of an agonist.

Partial agonists bind to receptors and induce only part of the changes in the receptors that are induced by agonists. The differences can be qualitative or quantitative. Thus, a

partial agonist may induce some of the conformation changes induced by agonists, but not others, or it may only induce certain changes to a limited extent.

Ligand-induced Conformational Changes

As described herein, the unliganded receptor is in a configuration that is either inactive, has some activity or has repressor activity. Binding of agonist ligands induces conformational changes in the receptor such that the receptor becomes more active, either to stimulate or repress the expression of genes. The receptors may also have non-genomic actions. Some of the known types of changes and/or the sequelae of these are listed herein.

Heat Shock Protein Binding

For many of the nuclear receptors ligand binding induces a dissociation of heat shock proteins such that the receptors can form dimers in most cases, after which the receptors bind to DNA and regulate transcription.

Nuclear receptors usually have heat shock protein binding domains that present a region for binding to the LBD and can be modulated by the binding of a ligand to the LBD. Consequently, an extended chemical moiety (or more) from the ligand that stabilizes the binding or contact of the heat shock protein binding domain with the LBD can be designed using the computational methods described herein to produce a partial agonist or antagonist. Typically such extended chemical moieties will extend past and away from the molecular recognition domain on the ligand and usually past the buried binding cavity of the ligand.

Dimerization and Heterodimerization

With the receptors that are associated with the hsp in the absence of the ligand, dissociation of the hsp results in dimerization of the receptors. Dimerization is due to receptor domains in both the DBD and the LBD. Although the main stimulus for dimerization is dissociation of the hsp, the ligand-induced conformational changes in the receptors may have an additional facilitative influence. With the receptors that are not associated with hsp in the absence of the ligand, particularly with the TR, ligand binding can affect the pattern of dimerization/heterodimerization. The influence depends on the DNA binding site context, and may also depend on the promoter context with respect to other

proteins that may interact with the receptors. A common pattern is to discourage monomer formation, with a resulting preference for heterodimer formation over dimer formation on DNA.

Nuclear receptor LBDs usually have dimerization domains that present a region for binding to another nuclear receptor and can be modulated by the binding of a ligand to the LBD. Consequently, an extended chemical moiety (or more) from the ligand that disrupts the binding or contact of the dimerization domain can be designed using the computational methods described herein to produce a partial agonist or antagonist. Typically such extended chemical moieties will extend past and away from the molecular recognition domain on the ligand and usually past the buried binding cavity of the ligand.

DNA Binding

In nuclear receptors that bind to hsp, the ligand-induced dissociation of hsp with consequent dimer formation allows, and therefore, promotes DNA binding. With receptors that are not associated (as in the absence of ligand), ligand binding tends to stimulate DNA binding of heterodimers and dimers, and to discourage monomer binding to DNA. However, ligand binding to TR, for example, tends to decrease dimer binding on certain DNA elements and has minimal to no effect on increasing heterodimer binding. With DNA containing only a single half site, the ligand tends to stimulate the receptor's binding to DNA. The effects are modest and depend on the nature of the DNA site and probably on the presence of other proteins that may interact with the receptors. Nuclear receptors usually have DBDs that present a region for binding to DNA and this binding can be modulated by the binding of a ligand to the LBD. Consequently, an extended chemical moiety (or more) from the ligand that disrupts the binding or contact of the DBD can be designed using the computational methods described herein to produce a partial agonist or antagonist. Typically such extended chemical moieties will extend past and away from the molecular recognition domain on the ligand and usually past the buried binding cavity of the ligand.

Repressor Binding

Receptors that are not associated with hsp in the absence of ligand frequently act as transcriptional repressors in the absence of the ligand. This appears to be due, in part, to

transcriptional repressor proteins that bind to the LBD of the receptors. Agonist binding induces a dissociation of these proteins from the receptors. This relieves the inhibition of transcription and allows the transcriptional transactivation functions of the receptors to become manifest.

Transcriptional Transactivation Functions

Ligand binding induces transcriptional activation functions in two basic ways. The first is through dissociation of the hsp from receptors. This dissociation, with consequent dimerization of the receptors and their binding to DNA or other proteins in the nuclear chromatin allows transcriptional regulatory properties of the receptors to be manifest. This may be especially true of such functions on the amino terminus of the receptors.

The second way is to alter the receptor to interact with other proteins involved in transcription. These could be proteins that interact directly or indirectly with elements of the proximal promoter or proteins of the proximal promoter. Alternatively, the interactions could be through other transcription factors that themselves interact directly or indirectly with proteins of the proximal promoter. Several different proteins have been described that bind to the receptors in a ligand-dependent manner. In addition, it is possible that in some cases, the ligand-induced conformational changes do not affect the binding of other proteins to the receptor, but do affect their abilities to regulate transcription.

Nuclear receptors or nuclear receptor LBDs usually have activation domains modulated in part by a co-activator/co-repressor system that coordinately functions to present a region for binding to DNA, and can be modulated by the binding of a ligand to the LBD. Consequently, an extended chemical moiety (or more) from the ligand that disrupts the binding or contact of the activation domain with co-activator and/or co-repressor can be designed using the computational methods described herein to produce a partial agonist or antagonist. For instance, an agonist can be designed and/or selected which (1) blocks binding and/or dissociates co-repressor, and/or (2) promotes binding and/or association of a co-activator. An antagonist can be designed which (1) promotes binding and/or association of co-repressor, and/or (2) promotes binding and/or association of co-activator. Ratios of agonists and antagonists may be used to modulate transcription of the gene of interest. Selection can be accomplised in binding assays that screen for ligands having the desired

agonist or antagonist properties, including such ligands which induce confomrational changes as decribed below. Suitable assays for such screening are described herein and in Shibata, H., *et al.* (*Recent Prog. Horm. Res. 52:*141-164 (1997)); Tagami, T., *et al. (Mol. Cell Biol. 17*(5):2642-2648 (1997)); Zhu, XG., *et al. (J. Biol. Chem. 272*(14):9048-9054 (1997)); Lin, B.C., *et al. (Mol. Cell Biol. 17*(10):6131-6138 (1997)); Kakizawa, T., *et al. (J. Biol. Chem. 272*(38):23799-23804 (1997)); and Chang, K. H., *et al. (Proc. Natl. Acad. Sci. USA 94*(17):9040-9045 (1997)). Typically such extended chemical moieties will extend past and away from the molecular recognition domain on the ligand and usually past the buried binding cavity of the ligand and in the direction of the activation domain, which is often a helix as seen in the three dimensional model shown in the **FIGURES** in two dimensions on paper or more conveniently on a computer screen.

Ligand-Induced Conformational Change

Plasma proteins bind hormones without undergoing a conformational change through a static binding pocket formed between monomers or domains. For example, the tetrameric thyroid-binding plasma protein transthyretin forms a solvent-accessible hormone-binding channel at the oligomer interface. The structure of the protein is unchanged upon binding hormone with respect to the appearance of a buried binding cavity with a ligand bound.

However, the structural role for a ligand bound to a nuclear receptor LBD, like rat TR-α LBD, predicts that the receptor would differ in the bound and unbound states. In the absence of hormone, the receptor would possess a cavity at its core, uncharacteristic of a globular protein. A ligand (e.g. hormone) completes the hydrophobic core of the active receptor after it binds to the nuclear receptor. Ligand binding by the receptor is a dynamic process, which regulates receptor function by inducing an altered conformation.

An exact description of the hormone-induced conformational changes requires comparison of the structures of the liganded and the unliganded TR. The structure of the unliganded human RXRα may substitute as a model for the unliganded TR. The rat TR-α LBD and human RXRα LBDs adopt a similar fold, and it is likely that the structural similarity extends to the conformational changes after ligand binding.

There are three major differences between the two structures, which indeed appear to be the result of ligand binding. First, the bound rat TR-α LBD structure is more compact,

with the hormone tightly packed within the hydrophobic core of the receptor. By contrast, the unliganded human RXRα LBD contains several internal hydrophobic cavities. The presence of such cavities is unusual in folded proteins, and is likely a reflection of the unliganded state of the receptor. Two of these cavities were proposed as possible binding sites for 9-cis retinoic acid, though these multiple sites only partly overlap with the single buried binding cavity observed in the liganded rat TR-α LBD.

The second difference involves H11 in the rat TR-α LBD, which contributes part of the hormone binding cavity. H11, continuous in the rat TR-α LBD, is broken at Cys 432 in the RXR, forming a loop between H10 and H11 in the hRXRα. This residue corresponds to His381 in the TR, which provides a hydrogen bond to the outer ring hydroxyl of the ligand. Furthermore, the hormone binding cavity occupied by ligand in the rat TR-α LBD is interrupted in the hRXRα by the same loop, forming an isolated hydrophobic pocket in the RXR with H6 and H7. In the bound rat TR-α LBD, the corresponding helices H7 and H8 are contiguous with the binding pocket, and enclose the hormone binding cavity from below.

The third difference between the two receptors is the position of the C-terminal activation domain. While the C-terminal activation domain forms α-helices in both receptors, the domain in the rat TR-α LBD follows a proline-rich turn, and lies against the receptor to contribute part of the binding cavity. In contrast, the activation domain in the unliganded hRXRα, is part of a longer helix which projects into the solvent.

These differences lead to a model for an alternate conformation of the TR LBD assumed in the absence of ligand. In the unliganded TR, the subdomain of the receptor surrounding the hormone binding cavity is loosely packed, with the binding cavity occluded by a partly unstructured H11 providing a partial core for the receptor.

Upon binding hormone, residues which form a coil in the unbound receptor engage the ligand, and continues H11. The ordering of H11 could unblock the hydrophobic cavity, allowing H7 and H8 to interact with hormone. The extended hydrophobic cavity then collapses around the hormone, generating the compact bound structure.

It is possible to predict ligand-induced conformational changes in the C-terminal activation domain that rely, in part, on an extended structure in the unliganded TR that repacks upon ligand binding. The ligand- induced conformation change can be subtle since the amino acid sequence of the rat TR-α in the turn (393-PTELFPP-399) significantly

reduces the propensity of the peptide chain of the rat TR-α to form an α-helix and therefore repacking can be accomplished with a minor change in volume.

After the ligand-induced conformational change occurs, it is likely that the conformation of the C-terminal activation domain in the bound structure changes packing compared to the unbound form of the receptor. Binding of the ligand improves the stability of the activation domain. The activation domain packs loosely even in the bound structure, as measured by the distribution of packing interactions for the entire LBD. The packing density for the activation domain, defined as the number of atoms within 4.5Å, is 1.5 standard deviations below the mean. For comparison, another surface helix, H1, is 0.5 standard deviations below the mean and the most poorly packed part of the structure, the irregular coil from residues Ile196-Asp206, is 2.0 standard deviations below the mean. Moreover, the majority of packing contacts for the C-terminal domain in the bound receptor are provided either by residues which interact with ligand, such as His381, or by the ligand itself. The conformation of these residues can be expected to be different in the bound and unbound receptors, and by extension the conformation of C-terminal activation domain which relies upon these interactions. Without the stabilization provided by a bound ligand, it is likely that the C-terminal activation domain is disordered prior to hormone binding.

The interrelation of ligand-induced conformational changes is evident as described herein. For example, His381 from H11 and Phe405 from H12 interact in the bound structure to provide a specific hydrogen bond to the phenolic hydroxyl. The ligand-induced changes which affect H11 and H12 are reinforcing, and lead to the formation of the compact, bound state.

Comparison of the TR-α and TR-β LBD structures shows similar packing of the helices when complexed with the ligand Triac.

COMPUTATIONAL METHODS USING THREE DIMENSIONAL MODELS AND EXTENSIONS OF LIGANDS

The three-dimensional structure of the liganded TR receptor is unprecedented, and will greatly aid in the development of new nuclear receptor synthetic ligands, such as thyroid receptor antagonists and improved agonists, especially those that bind selectively to one of the two TR isoforms (α or β). In addition, this receptor superfamily is overall well suited to

modern methods including three-dimensional structure elucidation and combinatorial chemistry such as those disclosed in EP 335 628, U.S. patent 5,463,564, which are incorporated herein by reference. Structure determination using X-ray crystallography is possible because of the solubility properties of the receptors. Computer programs that use crystallography data when practicing the present invention will enable the rational design of ligand to these receptors. Programs such as RASMOL can be used with the atomic coordinates from crystals generated by practicing the invention or used to practice the invention by generating three dimensional models and/or determining the structures involved in ligand binding. Computer programs such as INSIGHT and GRASP allow for further manipulation and the ability to introduce new structures. In addition, high throughput binding and bioactivity assays can be devised using purified recombinant protein and modern reporter gene transcription assays described herein and known in the art in order to refine the activity of a CDL.

Generally the computational method of designing a nuclear receptor synthetic ligand comprises two steps:

1) determining which amino acid or amino acids of a nuclear receptor LBD interacts with a first chemical moiety (at least one) of the ligand using a three dimensional model of a crystallized protein comprising a nuclear receptor LBD with a bound ligand, and

2) selecting a chemical modification (at least one) of the first chemical moiety to produce a second chemical moiety with a structure to either decrease or increase an interaction between the interacting amino acid and the second chemical moiety compared to the interaction between the interacting amino acid and the first chemical moiety.

As shown herein, interacting amino acids form contacts with the ligand and the center of the atoms of the interacting amino acids are usually 2 to 4 angstroms away from the center of the atoms of the ligand. Generally these distances are determined by computer as discussed herein and in McRee 1993, however distances can be determined manually once the three dimensional model is made. Examples of interacting amino acids are described in Appendix 2. See also Wagner *et al.*, *Nature* 378(6558):670-697 (1995) for stereochemical figures of three dimensional models. More commonly, the atoms of the ligand and the atoms of interacting amino acids are 3 to 4 angstroms apart. The invention can be practiced by repeating steps 1 and 2 to refine the fit of the ligand to the LBD and to determine a better

ligand, such as an agonist. As shown in the **FIGURES** the three dimensional model of TR can be represented in two dimensions to determine which amino acids contact the ligand and to select a position on the ligand for chemical modification and changing the interaction with a particular amino acid compared to that before chemical modification. Structural comparison of LBD isoforms complexed with the same or similar ligand permit identification of fiducial and adjustable amino acids that can be exploited in designing isoform-specific ligands through chemical modification. "Fiducial" refers to amino acids that form rigid features of the ligand binding cavity. "Adjustable" refers to amino acids that form less rigid features of the ligand binding cavity. The chemical modification may be made using a computer, manually using a two dimensional representation of the three dimensional model or by chemically synthesizing the ligand. The three dimensional model may be made using Appendix 2 and the FIGURES. As an additional step, the three dimensional model may be made using atomic coordinates of nuclear receptor LBDs from crystallized protein as known in the art, see McRee 1993 referenced herein.

The ligand can also interact with distant amino acids after chemical modification of the ligand to create a new ligand. Distant amino acids are generally not in contact with the ligand before chemical modification. A chemical modification can change the structure of the ligand to make as new ligand that interacts with a distant amino acid usually at least 4.5 angstroms away from the ligand. Often distant amino acids will not line the surface of the binding cavity for the ligand, as they are too far away from the ligand to be part of a pocket or surface of the binding cavity.

The interaction between an atom of a LBD amino acid and an atom of an LBD ligand can be made by any force or attraction described in nature. Usually the interaction between the atom of the amino acid and the ligand will be the result of a hydrogen bonding interaction, charge interaction, hydrophobic interaction, van der waals interaction or dipole interaction. In the case of the hydrophobic interaction it is recognized that this is not a per se interaction between the amino acid and ligand, but rather the usual result, in part, of the repulsion of water or other hydrophilic group from a hydrophobic surface. Reduction or enhancment of the interaction of the LBD and a ligand can be measured by standard binding procedures, calculating or testing binding energies, computationally or using thermodynamic or kinetic methods as known in the art.

Chemical modifications will often enhance or reduce interactions of an atom of a LBD amino acid and an atom of an LBD ligand. Steric hinderance will be a common means of changing the interaction of the LBD binding cavity with the activation domain. Chemical modifications are preferably introduced at C-H, C- and C-OH position in ligands, where the carbon is part of the ligand structure which remains the same after modification is complete. In the case of C-H, C could have 1, 2 or 3 hydrogens, but usually only one hydrogen will be replaced. The H or OH are removed after modification is complete and replaced with the desired chemical moiety.

Because the thyroid receptor is a member of the larger superfamily of hormone-binding nuclear receptors, the rules for agonist and antagonist development will be recognized by one skilled in the art as useful in designing ligands to the entire superfamily. Examining the structures of known agonists and antagonists of the estrogen and androgen receptors supports the generality of antagonist mechanism of action as shown in **FIG. 10**.

The overall folding of the receptor based on a comparison of the reported structure of the unliganded RXR and with amino acid sequences of other superfamily members reveals that the overall folding of receptors of the superfamily is similar. Thus, it is predicted from the structure that there is a general pattern of folding of the nuclear receptor around the agonist or antagonist ligand.

The three dimensional structure of a nuclear receptor with a ligand bound leads to the nonobvious observation that a nuclear receptor folds around agonist ligands, as the binding cavity fits the agonist, especially the agonist's molecular recognition domain, and antagonists commonly have chemical structures that extend beyond the ligand, especially the agonist, and would prohibit folding of the receptor around the ligand to form a buried binding cavity or other groups that have the same effect. The location of the extension could affect the folding in various ways as indicated by the structure. Such extensions on antagonists are shown in **FIG. 10** for various receptors and compared to the corresponding agonist.

For example, an extension towards the carboxy-terminal activation helix affects the packing/folding of this helix into the body of the receptor. This in turn can affect the ability of this portion of the nuclear receptor to interact with other proteins or other portions of the receptor, including transcriptional transactivation functions on the opposite end of the linear receptor, or the receptor's amino terminus that may interact directly or indirectly with the

carboxy-terminal transactivation domain (including helix 12). Extensions in this direction can also affect the packing of helix 11 of TR (or its analogous helix in nuclear receptors) into the body of the receptor and selectively affect dimerization and heterodimerization of receptors. An extension pointing towards helix 1 can affect the relationship of the DNA binding domain and hinge regions of the receptors with the ligand binding domain and selectively or in addition affect the receptors' binding to DNA and/or interactions of receptors with proteins that interact with this region of the receptor. Other extensions towards helix 11 can be made to affect the packing of this helix and helices 1 and 10 and thereby homo- and hetero-dimerization. Such chemical modifications can be assessed using the computational methods described herein. It is also possible that, in some cases, extensions may protrude through the receptor that is otherwise completely or incompletely folded around the ligand. Such protruding extensions could present a steric blockade to interactions with co-activators or other proteins.

The three dimensional structure with the ligand buried in the binding cavity immediately offers a simple description of a nuclear receptor that has a binding cavity that contains hinges and a lid, composed of one or more structural elements, that move to accommodate and surround the ligand. The ligand to TR can be modified on specific sites with specific classes of chemical groups that will serve to leave the lid and hinge region in open, partially open or closed states to achieve partial agonist or antagonist functions. In these states, the biological response of the TR is different and so the structure can be used to design particular compounds with desired effects.

Knowledge of the three-dimensional structure of the TR-T_3 complex leads to a general model for agonist and antagonist design. An important novel feature of the structural data is the fact that the T_3 ligand is completely buried within the central hydrophobic core of the protein. Other ligand-receptor complexes belonging to the nuclear receptor superfamily will have a similarly buried ligand binding site and therefore this model will be useful for agonist/antagonist design for the entire superfamily.

When design of an antagonist is desired, one needs either to preserve the important binding contacts of natural hormone agonist while incorporating an "extension group" that interferes with the normal operation of the ligand-receptor complex or to generate the requisite binding affinity through the interactions of the extensions with receptor domains.

The model applied to antagonist design and described herein is called the "Extension Model." Antagonist compounds for nuclear receptors should contain the same or similar groups that facilitate high-affinity binding to the receptor, and in addition, such compounds should contain a side chain which may be large and/or polar. This side chain could be an actual extension, giving it bulk, or it could be a side group with a charge function that differs from the agonist ligand. For example, substitution of a CH_3 for CH_2OH at the 21-position, and alteration at the 11-position from an OH group to a keto group of cortisol generates glucocorticoid antagonist activity (Robsseau, G.G., *et. al.*, *J. Mol. Biol. 67*:99-115 (1972)). However, in most cases effective antagonists have more bulky extensions. Thus, the antiglucocorticoid (and antiprogestin) RU486 contains a bulky side group at the 11-position (Horwitz, K.B. *Endocrine Rev. 13*:146-163 (1992)). The antagonist compound will then bind within the buried ligand binding site of the receptor with reasonably high affinity (100 nM), but the extension function will prevent the receptor-ligand complex from adopting the necessary conformation needed for transcription factor function. The antagonism (which could be in an agonist or antagonist) may manifest itself at the molecular level in a number of ways, including by preventing receptor homo/heterodimer formation at the HRE, by preventing coactivator binding to receptor monomers, homodimers or homo/heterodimers, or by a combination of these effects which otherwise prevent transcription of hormone responsive genes mediated by ligand-induced effects on the HRE. There are several antagonist compounds for nuclear receptors in the prior art (see also Horwitz, K.B., *Endocrine Rev. 13*:146-163 (1992), Raunnaud J.P. *et. al.*, *J. Steroid Biochem. 25*:811-833 (1986), Keiel S., *et. al.*, *Mol. Cell. Biol. 14*:287-298 (1994) whose antagonist function can be explained by the extension hypothesis. These compounds are shown in **FIG. 10** along with their agonist counterparts. Each of these antagonists contains a large extension group attached to an agonist or agonist analogue core structure. Importantly, these antagonist compounds were discovered by chance and not designed with a structure-function hypothesis such as the extension principle.

One method of design of a thyroid antagonist using the extension hypothesis is provided below as a teaching example. The three-dimensional structure of the TR-α Dimit complex combined with structure-activity data published in the prior art, especially those reference herein, can be used to establish the following ligand-receptor interactions which are

most critical for high-affinity ligand binding. A physical picture of these interactions is shown in **FIG. 6**. The figure describes the isolated essential contacts for ligand binding. Because the ligand is buried in the center of the receptor, the structural spacing between these isolated interactions is also important. Thus, our present knowledge of this system dictates that, for this example, a newly designed ligand for the receptor must contain a thyronine structural skeleton, or two substituted aryl groups joined by a one-atom spacer.

The general structure for an antagonist designed by the extension hypothesis is exemplified in the following general description of the substituents of a TR antagonist (referring to Formula 1): R1 can have anionic groups such as a carboxylate, phosphonate, phosphate, sulfate or sulfite and is connected to the ring with a 0 to 3 atom linker, comprising one or more C, O, N, S atoms, and preferably a 2 carbon linker. Such R1 can be optionally substituted with an amine (e.g. -NH2). R3 and R5 are small hydrophobic groups such as -Br, -I, or -CH3. R3 and R5 can be the same substituents or different. R_3' can be a hydrophobic group that may be larger than those of R3 and R5, such as -I, -CH3, isopropyl, -phenyl, -benzyl, 5 and 6 ring heterocycles. R_4' is a group that can participate in a hydrogen bond as either a donor or acceptor. Such groups include -OH, -NH_2, and -SH. R_5' is an important extension group that makes this compound an antagonist. R_5' can be a long chain alkyl (e.g. 1 to 9 carbons, straight chain or branched), aryl (benzyl, phenyl and substituted benzyl and phenyl rings (e.g with halogen, alkyl (1 and 5 carbons) and optionally connected to the ring by a -CH2-), heterocycle (e.g. 5 or 6 atoms, preferably 5 carbons and 1 nitrogen, or five carbons), which can optionally include polar (e.g. -OH, -NH_2, and -SH), cationic (e.g. -NH3, N(CH)3), or anionic (carboxylate, phosphonate, phosphate or sulfate) groups. R_5' can also be a polar (e.g. -OH, -NH_2, and -SH), cationic (e.g. -NH3, -N(CH3)3), and anionic (carboxylate, phosphonate, phosphate or sulfate) groups. X is the spacer group that appropriately positions the two aromatic rings. This group is usually a one-atom spacer, such as O, S, SO, SO2, NH, NZ where Z is an alkyl, CH2, CHOH, CO, C(CH3)OH, and C(CH3)(CH3). X also may be NR_7, CHR_7, CR_7, R_7, where R_7, is an alkyl, aryl or 5- or 6-membered heterocyclic aromatic. R2, R6, R2' and R6' can be -F, and -Cl and are preferably H.

A TR ligand can also be described as a substituted phenylated 3,5 diiodo tyrosine with substituted R5' and R3' groups. R5' can be a long chain alkyl (e.g. 4 to 9 carbons, straight

chain or branched), aryl (benzyl, phenyl and substituted benzyl and phenyl rings (e.g with halogen, alkyl (1 and 5 carbons) and optionally connected to the ring by a -CH2-), heterocycle (e.g. 5 or 6 atoms, preferably 5 carbons and 1 nitrogen, or five carbons), which can optionally include polar (e.g. -OH, $-NH_2$, and -SH), cationic (e.g. -NH3, N(CH)3), or anionic (carboxylate, phosphonate, phosphate or sulfate) groups. R5' can also be a polar (e.g. -OH, $-NH_2$, and -SH), cationic (e.g. -NH3, N(CH)3), and anionic (carboxylate, phosphonate, phosphate or sulfate) groups. R3' can be -IsoPr, halogen, -CH3, alkyl (1 to 6 carbons) or aryl (benzyl, phenyl and substituted benzyl and phenyl rings (e.g with halogen, alkyl (1 and 5 carbons) and optionally connected to the ring by a -CH2-), heterocycle (e.g. 5 or 6 atoms, preferably 5 carbons and 1 nitrogen, or five carbons), which can optionally include polar (e.g. -OH, $-NH_2$, and -SH), cationic (e.g. -NH3, N(CH)3), or anionic (carboxylate, phosphonate, phosphate or sulfate) groups.

A TR antagonist can also be a modified T_3 agonist (having a biphenyl structure) wherein R_5' is alkyl, aryl, 5- or 6-membered heterocyclic aromatic, heteroalkyl, heteroaryl, arylalkyl, heteroaryl alkyl, polyaromatic, polyheteroaromatic, polar or charged groups, wherein said R_5' may be substituted with polar or charged groups. The R5' groups are defined, as described herein.

Using these methods the ligands of this example preferably have the following properties:

1. The compounds should bind to the TR with high affinity (for example 100 nM).
2. The compounds should bind the receptor in the same basic orientation as the natural hormone.
3. The extension group R5' should project toward the activation helix (C-terminal helix) of the receptor.
4. The appropriate substituent at R5' should perturb the activation helix from its optimal local structure needed for mediating transcription.

Antagonists may also be designed with multiple extensions in order to block more than one aspect of the folding at any time.

TR ligands (e.g. super agonists) can be designed (and synthesized) to enhance the interaction of at least one amino acid with at least one chemical moiety on the ligand's molecular recognition domain. One method is to enhance the charge and polar interactions

by replacing the carboxylate of T_3 (R1 position) with phosphonate, phosphate, sulfate or sulfite. This enhances the interaction with Arg 262, Arg 266 and Arg 228. The interaction of at least one amino acid with at least one chemical moiety on the ligand's molecular recognition domain can also be enhanced by increasing the size of R1 group to fill the space occupied by water when Dimit is bound (referring to R1). Preferably the group has a complementary charge and hydrophobicity to the binding cavity.

Another way of improving the interaction of at least one amino acid with at least one chemical moiety on the ligand's molecular recognition domain is to restrict the conformation of the dihedral angle between the two phenyl rings of the thyronine ligand in solution. In solution the planes of two phenyl rings are orthogonal where the dihedral angle is 90°. In the TR Dimit structure, the dihedral angle is close to 60°. A TR ligand design that fixes the angle between the two phenyl rings will lead to tighter binding. Such a ligand may be made by connecting the R6' and the R5 positions of a thyronine or a substituted thyronine-like biphenyl. The size of the cyclic connection can fix the angle between the two phenyl rings. Referring specifically to Formula 1, the following cyclic modifications are preferred: 1) R_5 is connected to R_6', 2) R_3 is connected to R_2' or 3) R_5 is connected to R_6' and R_3 is connected to R2'. The connections can be made by an alkyl or heteroalkyl chain having between 1 to 6 atoms and preferably from 2 to 4 carbon atoms or other atoms. Any position of the heteroalkyl chain can be N, O, P or S. The S and P heteroatoms along said heteroalkyl chain are in any of their possible oxidative states. The N heteroatom or any carbon along the alkyl or heteroalkyl chain may have one or more Z substituents, wherein Z is alkyl, heteroalkyl, aryl, heteroaryl, 5- or 6-membered heterocyclic aromatic. These compounds can be claimed with the proviso that Formula 1 does not include any prior art compound as of the priority filing date of this application.

The interaction of at least one amino acid with at least one chemical moiety on the ligand's molecular recognition domain can also be enhanced by selecting a chemical modification that fills the unfilled space between a TR ligand and the LBD in the area of the bridging oxygen (such as in T3, Triac or Dimit). Thus, a slighter larger moiety that replaces the ether oxygen can enhance binding. Such a linker may be a mono- or geminal-disubstituted carbon group. A group approximately the same size as oxygen but with greater

hydrophobicity is preferred as well as small, hydrophobic groups for the disubstituted carbon.

Compounds of Formula I or derivatives thereof that modulate TR activity also may be designed and selected to interact with a conformationally constrained structural feature of a TR LBD that is conserved among TR LBD isoforms to increase TR-specific selectivity. Conserved structural features of a TR LBD include residues found in equivalent positions of TR LBD isoforms which interact with a conserved structural feature of a compound comprising the biphenyl scaffold (ϕ-X-ϕ) or a single phenyl scaffold (ϕ-X or X-ϕ) of Formula I. Conformationally constrained structural features of a TR LBD include residues that have their natural flexible conformations fixed by various geometric and physical-chemical constraints, such as local backbone, local side chain, and topological constraints. These types of constraints are exploited to restrict positioning of atoms involved in receptor-ligand recognition and binding. For example, comparison of atomic models of TR LBD isoforms bound to thyronine and thyronine-like ligands reveal that certain residues which contact the ligands are restricted to particular topological shapes and angles of rotation about bonds. These include Met259, Leu276, Leu292, His381, Gly290, Ile221, and Phe401 of TR-α. The corresponding positions in TR-β include Met313, Leu330, Leu346, His435, Gly344, Ile275 and Phe455, respectively.

Selectivity imparted by conformationally constrained features of both the receptor and compound are of particular interest. For example, compounds of Formula I comprising constrained cyclic carbons and substituent groups that interact with a constrained feature of a TR LBD can be exploited to further increase binding specificity while reducing the potential for cross-over interaction with other receptors. These include hydrophobic and/or hydrophilic contacts between constrained residues of a TR LBD and atomic groups of the following constituents of the compound in reference to Formula I: (i) the biphenyl rings; (ii) the R3-substituent; (iii) the R3'-substituent; and (iv) the R4'-substituent.

For example, contacts to the phenyl moiety comprising the R1, R2, R3, R5 and R6 substituents, i.e., the ring proximal to the polar pocket (the "inner ring"), include a cycle carbon atom that interacts with an atom of a hydrophobic residue of a TR LBD, such as a carbon and oxygen atom of Met259 and a carbon atom of Leu276 of TR-α, or Met313 and Leu330 of TR-β, where the cycle carbon is about 3.0 to 4.0A from the atom of the

hydrophobic group. For example, comparison of TR-α complexed with T3 and TR-β complexed with GC-1 reveals the following conserved inner ring contacts:

Ligand	TR LBD		
T3/Atom	TR-α Residue	Atom	Distance
C11	Met259	C	3.95
C11	Met259	O	3.59
C11	Met259	CB	3.77
C7	Leu276	CD2	3.80
C9	Leu276	CD2	3.70
GC1/Atom	TR-β Residue	Atom	Distance
C11	Met313	C	3.85
C11	Met313	O	3.41
C11	Met313	CB	3.79
C7	Leu330	CD2	3.56
C9	Leu330	CD2	3.63

Contacts to the phenyl moiety comprising the R2', R3', R4', R5' and R6' substituents, i.e., the ring distal to the polar pocket (the "outer ring"), include a cyclic carbon atom that interacts with an atom of a hydrophobic residue of a TR LBD, such as a carbon atom of Leu292 of TR-α, or Leu346 of TR-β, where the cyclic carbon atom is about 3.0 to 4.0A from the atom of the hydrophobic residue. For example, comparison of TR-α complexed with T3 and TR-β complexed with GC-1 reveals the following conserved outer ring contacts:

Ligand	TR LBD		
T3/Atom	TR-α Residue	Atom	Distance
C6	Leu292	CD2	3.58
C8	Leu292	CD2	3.50
GC1/Atom	TR-β Residue	Atom	Distance
C6	Leu346	CD2	3.77
C8	Leu346	CD2	3.80

Contacts to the R3-substituent include an atom that interacts with a carbon atom of a hydrophobic residue of a TR LBD, such as Ile221 of TR-α, or Ile275 of TR-β, where the R3-substituent atom is about 3.0 to 4.0A from the carbon atom of the hydrophobic residue.

For example, comparison of TR-α complexed with T3 and TR-β complexed with GC-1 reveals the following conserved R3-substituent contacts:

Ligand	TR LBD		
T3/Atom	TR-α Residue	Atom	Distance
I1	Ile221	CG1	4.01
GC1/Atom	TR-β Residue	Atom	Distance
C19	Ile275	CG1	3.98

Contacts to the R3'-substituent include an atom that interacts with an atom of a hydrophobic or hydrophilic residue of a TR LBD, such as an oxygen atom of Gly290 of TR-α, or Gly344 of TR-β, where the R3'-substituent atom is about 3.0 to 4.0A from the atom of the hydrophobic or hydrophilic residue. For example, comparison of TR-α complexed with T3 and TR-β complexed with GC-1 reveals the following conserved R4'-substituent, phenolic hydroxyl contacts:

Ligand	TR LBD		
T3/Atom	TR-α Residue	Atom	Distance
I2	Gly290	O	3.50
GC1/Atom	TR-β Residue	Atom	Distance
C18	Gly344	O	3.60

Contacts to the R4'-substituent comprising a phenolic hydroxyl include carbon and oxygen atoms that interact with a hydrophobic or hydrophilic residue of a TR LBD, such as a carbon and nitrogen atom of His381 of TR-α, or His435 of TR-β, where the R4'-substituent atom is about 2.0 to 4.0A from an atom of the hydrophobic or hydrophilic residue. For example, comparison of TR-α complexed with T3 and TR-β complexed with GC-1 reveals the following conserved R4'-substituent, phenolic hydroxyl contacts:

Ligand	TR LBD		
T3/Atom	TR-α Residue	Atom	Distance
C10	His381	CD2	3.97
O1	His381	CD2	3.39
O1	His381	CE1	3.82
C8	His381	NE2	3.47
C10	His381	NE2	3.55
O1	His381	NE2	2.70
GC1/Atom	TR-β Residue	Atom	Distance
C10	His435	CD2	3.89
O1	His435	CD2	3.64
O1	His435	CE1	3.79
C8	His435	NE2	3.44
C10	His435	NE2	3.33
O1	His435	NE2	2.77

Contacts to the R4'-substituent also may include an atom that interacts with a carbon atom of a hydrophobic residue of a TR LBD, such as Phe401 of TR-α, or Phe455 of TR-β, for defining agonist activity, i.e., proper presentation of helix-12 (H12) of the TR LBD following ligand binding. The R4'-substituent atom is about 3.0 to 4.0A from the carbon atom of the hydrophobic group. For example, comparison of TR-α complexed with T3 and TR-β complexed with GC-1 reveals the following conserved R4'-substituent contacts:

Ligand	TR LBD		
T3/Atom	TR-α Residue	Atom	Distance
O1	Phe401	CE1	3.52
O1	Phe401	CZ	3.32
GC1/Atom	TR-β Residue	Atom	Distance
O1	Phe455	CE1	3.40
O1	Phe455	CZ	3.22

Comparison of atomic models of TR LBD isoforms complexed with the same and/or different ligands therefore facilitates the identification of new compounds that fit spacially and preferentially into a TR LBD. Modeling, comparison of TR-ligand overlays, and comparison of TR LBD isoforms also permit identification of conformationally conserved structural features of TR LBD/ligand contacts. Exploiting conformational constraints of the LBD-ligand interaction identified by such methods therefore improves the design and

identification of new compounds having increased selectivity for binding a particular type of nuclear receptor, such as TR.

TR-α AND TR-β SELECTIVITY FOR THE THYROID HORMONE RECEPTOR

Using the method described herein ligands can be designed that selectively bind to the alpha more than the beta TR or vice versa. The X-ray crystallographic structure of the rat TR-α LBD provides insight into design of such ligands.

The three dimensional structure reveals that the major difference between the TR-α and TR-β in the ligand binding cavity resides in amino acid Ser 277 (with the side group -CH2OH) in the rat TR-α and whose corresponding residue is 331, asparagine (with the side group -CH2CONH2), in the human TR-β. The side chain in human TR-β is larger, charged and has a different hydrogen bonding potential, which would allow the synthesis of compounds that discriminate between this difference. The Ser277 (Asn331 in TR-β) forms part of the polar pocket of the TR LBD, indicating that for TR-α versus TR-β discrimination, ligands can be designed to contain chemical modification of the R1-substitutent with reference to Formula I that exploit this difference.

For example, in the complex of TR-α with Triac, Ser277 does not participate in ligand binding. The absence of a role for Ser277 (Asn331 in beta) is consistent with the equal affinity of Triac for the alpha and beta isoforms, and indirectly supports the contention that alpha/beta selectivity resides in the amino acid substitution Ser277 to Asn331 and its interaction with Arg228. The effect of the amino acid substitution is further evident when the interactions of Asn331 and Arg282 in the structures of the TR-β LBD complexed with GC-1 or Triac are compared with those of Ser277 and Arg228 in the TR-α LBD. In the complex with GC-1, Asn331 forms a hydrogen bond to Arg282, which in turn forms a hydrogen bond with the carboxylate of GC-1, a pattern that resembles the interactions of Ser277 and Arg228 in the complexes of the TR-α LBD complexed with T_3 or Triac. However, in the complex of TR-β with Triac, Arg282 rotates away from Asn331 and the ligand, instead forming hydrogen bonds to residues Thr287 and Asp291 of H3. Therefore, differences exist between the two isoforms in the conformation of the polar pocket, depending on the nature of the ligand R1-substitutent, indicating that certain substituents may interact preferentially with the conformation of a given isoform.

Comparing overlays of various ligands bound to the TR-α versus TR-β LBDs shows the positioning of the ligand to be very similar. Surprisingly, comparison of the volume and area for the TR-α and TR-β LBDs bound by the same or different ligands unexpectedly shows that the cubic space or volume available for accommodating ligand binding by the TR-β LBD (645 $\pm$ 28.28 Å^3) is larger and more flexable than that of the TR-α LBD (596.25 $\pm$ 7.97 Å^3) (Table 1). The volume of the ligand binding cavity for TR-α varies over a narrow range of about 8+, with a maximum difference of about 16+. In contrast, the volume of the ligand binding cavity for TR-β differs by nearly 40+ between the complexes with GC-1 and Triac. There also is a difference in the volume of the ligand binding cavity when comparing the same ligand bound to TR-α and TR-β. For example, TR-α and TR-β complexed with Triac differ in LBD volume by about 36 Å^3. Comparison of TR-α and TR-β bound to Dimit and GC-1, respectively, which ligands have similar volume/area and superpositioned architecture, show that the difference in LBD volume is about 75 Å^3. These differences are attributed primarily to variable movement and interaction of side chain groups with ligand substitutents of the phenyl moiety (ϕ) of the biphenyl scaffold (ϕ-X-ϕ) located proximal to the polar pocket, e.g., R1-substituents in reference to Formula I. In contrast, the volume available in the hydrophobic pocket for both the TR-α and TR-β LBDs is substantially the same. For example, binding of Triac to the TR-β LBD displaces the side chain of Arg 282 providing approximately 60 Å^3 in the polar pocket cavity, exposing the polar pocket to bulk solvent exchange. For GC1 bound to the TR-β LBD, approximately 14 Å^3 is due to side chain motion of Met310, and approximately 44 Å^3 is due to side chain motion of Arg320, the combination of which increases the size of the polar pocket in the TR-β LBD. This extra pliability also may explain the absence of ordered water in the polar pocket of TR-β LBD bound to Triac or GC-1, which is in contrast to the ordered water found in the polar pocket of TR-α LBD bound to Dimit, IpBr2 or T3.

Table 1*

	rTR-α			
	Dimit	Triac	IpBr2	T3
TR LBD (volÅ^3/areaÅ^2)	590/456	589/440	601/474	605/472
Ligand (volÅ^3/areaÅ^2)	303/314	333/326	326/330	355/346
Complementarity	0.65	0.68	0.66	0.71

	hTR-β	
	GC-1	Triac
TR LBD (volÅ^3/areaÅ^2)	665/575	625/474
Ligand (volÅ^3/areaÅ^2)	294/310	333/326
Complementarity	0.61	0.67

*TR LBD volume and area are reported in Angstroms measured by GRASP. Complementarity is determined as defined in Lawrence *et al.*, *J. Mol. Biol. 234*:946-950 (1993).

Residue Ser277 in TR-α and the corresponding residue Asn331 of TR-β also contribute to the volumetric differences observed in the polar pockets of these two TR isoforms. And substitution of the Asn331 of hTR-β with serine has the affect of modifying ligand binding affinity of TR-β so that it resembles that of TR-α (See Example 5). Taken together, differences in hydrogen bonding of atoms of the side chain group of Ser277 in TR-α and Asp331 in TR-β extending from the equivalent backbone position in these TR LBDs and the more restricted polar pocket of the TR-α LBD further supports the concept of designing TR LBD isoform-specific ligands having substitutents that fit spacially and preferentially into the polar pocket of either the TR-α or TR-β LBDs. Exploitation of this difference provides an additional means for computational design of isoform-specific TR agonists and antagonists.

In terms of ligand design, these differences mean that for β-selective ligands, some or all of the following differences should be exploited:

1. The presence of a larger side chain asparagine.
2. The ability of the carbonyl group on the side chain to provide a strong hydrogen bond acceptor.
3. The ability of the amido group on the side chain to provide a two hydrogen bond donors.
4. Adjustment of polarity to reorganize the trapped water in the T3 pocket.

5. Greater size and flexibility of the polar pocket.

In terms of pharmaceutical design, these differences mean that for α-selective ligands, some or all of the following differences should be exploited:

1. The presence of a smaller side group.
2. The ability of the hydroxyl on the $-CH_2OH$ side group carbonyl group on the side chain to provide a weak hydrogen donor.
3. Adjustment of polarity to reorganize the trapped water in the T3 pocket.
4. Smaller size and limited flexibility of the polar pocket.

In both cases these differences can be exploited in a number of ways. For example, they can also be used with a software set for construction of novel organic molecules such as LUDI from Biosym-MSI. An example of designing TR-β selective ligands is increasing the polarity of a ligand substituent located in the polar pocket of a TR LBD through addition of one or more ligand groups having a formal negative charge and/or negative dipole charge that interacts with a formal positive charge and/or positive dipole charge of a group in the polar pocket of the LBD. This exploits preferential interactions, such as with the additional positive charge contributed by Asn 331 in TR-β. Another example of a TR-β selective ligand is one that comprises one or more groups which fit spacially into the TR-β LBD polar pocket. This exploits spacial differences between TR LBD isoforms, such as the larger and more flexible polar pocket of TR-β.

METHODS OF TREATMENT

The compounds of Formula 1 can be useful in medical treatments and exhibit biological activity which can be demonstrated in the following tests:

(i) the induction of mitochondrial α-glycerophosphate dehydrogenase (GPDH:EC 1.1.99.5). This assay is particularly useful since in certain species e.g. rats it is induced specifically by thyroid hormones and thyromimetics in a close-related manner in responsive tissues e.g. liver, kidney and the heart (Westerfield, W.W., Richert, D.A. and Ruegamer, W.R., *Endocrinology* (1965) *77*:802). The assay allows direct measurement in rates of a thyroid hormone-like effect of compounds and in particular allows measurement of the direct thyroid hormone-like effect on the heart. Other measurements included parameters such as

heart rate and cardiac enzymes including Ca^{++} ATPase, Na^{++}/K^{+} ATPase, myosin isoforms and specific liver enzymes;

(ii) the elevation of basal metabolic rate as measured by the increase in whole body oxygen consumption (see e.g., Barker *et al.*, *Ann. N. Y. Acad. Sci.*, (1960) *86*:545-562);

(iii) the stimulation of the rate of beating of atria isolated from animals previously dosed with thyromimetrics (see e.g., Stephan *et al.*, *Biochem. Pharmacol.* (1992) *13*:1969-1974; Yokoyama *et al.*, *J. Med. Chem.*, (1995) *38*:695-707);

(iv) the change in total plasma cholesterol levels as determined using a cholesterol oxidase kit (for example, the Merck CHOD iodine colorimetric kit. see also, Stephan *et al.* (1992));

(v) the measurement of LDL (low density lipoprotein) and HDL (high density lipoprotein) cholesterol in lipoprotein fractions separated by ultracentrifugation; and p (vi) the change in total plasma triglyceride levels as determined using enzymatic color tests, for example the Merck System GPO-PAP method.

The compounds of Formula 1 can be found to exhibit selective thyromimetic activity in these tests,

(a) by increasing the metabolic rate of test animals, and raising hepatic GPDH levels at doses which do not significantly modify cardiac GPDH levels.

(b) by lowering plasma cholesterol and triglyceride levels, and the ratio of LDL to HDL cholesterol at doses which do not significantly modify cardiac GPDH levels.

The compounds of Formula 1 may therefore be used in therapy, in the treatment of conditions which can be alleviated by compounds which selectively mimic the effects of thyroid hormones in certain tissues whilst having little or no direct thyromimetic effect on the heart. For example, compounds of Formula 1 which raise hepatic GPDH levels and metabolic rate at doses which do not significantly modify cardiac GPDH levels are indicated in the treatment of obesity.

Agonists of Formula 1 will lower total plasma cholesterol, the ratio of LDL-cholesterol to HDL-cholesterol and triglyceride levels at doses which do not significantly modify cardiac GPDH levels are indicated for use as general antihyperlipidaemic (antihyperlipoproteinaemic) agents i.e. in the treatment of patients having elevated plasma

lipid (cholesterol and triglyceride) levels. In addition, in view of this effect on plasma cholesterol and triglyceride, they are also indicated for use as specific anti-hypercholesterolemic and anti-hypertriglyceridaemic agents.

Patients having elevated plasma lipid levels are considered at risk of developing coronary heart disease or other manifestations of atherosclerosis as a result of their high plasma cholesterol and/or triglyceride concentrations. Further, since LDL-cholesterol is believed to be the lipoprotein which induces atherosclerosis, and HDL-cholesterol believed to transport cholesterol from blood vessel walls to the liver and to prevent the build up of atherosclerotic plaque, anti-hyperlipidemic agents which lower the ratio of LDL-cholesterol to HDL cholesterol are indicated as anti-atherosclerotic agents, herein incorporated by reference U.S. patents 4,826,876 and 5,466,861.

The present invention also provides a method of producing selective thyromimetic activity in certain tissues except the heart which comprises administering to an animal in need thereof an effective amount to produce said activity of a compound of Formula 1 or a pharmaceutically acceptable salt thereof.

The present invention also relates to a method of lowering plasma lipid levels and a method of lowering the ratio of LDL-cholesterol to HDL-cholesterol levels by suitably administering a compound of this invention or a pharmaceutically acceptable sale thereof.

In addition, compounds of Formula 1 may be indicated in thyroid hormone replacement therapy in patients with compromised cardiac function.

In therapeutic use the compounds of the present invention are usually administered in a standard pharmaceutical composition.

The present invention therefore provides in a further aspect pharmaceutical compositions comprising a compound of Formula 1 or à pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier. Such compositions include those suitable for oral, parenteral or rectal administration.

PHARMACEUTICAL COMPOSITIONS

Compounds of Formula 1 and their pharmaceutically acceptable salts which are active when given orally can be formulated as liquids for example syrups, suspensions or emulsions, tablets, capsules and lozenges.

A liquid composition will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s), for example ethanol, glycerine, sorbitol, non-aqueous solvent such as polyethylene glycol, oils or water, with a suspending agent, preservative, surfactant, wetting agent, flavoring or coloring agent. Alternatively, a liquid formulation can be prepared from a reconstitutable powder.

For example a powder containing active compound, suspending agent, sucrose and a sweetener can be reconstituted with water to form a suspension; and a syrup can be prepared from a powder containing active ingredient, sucrose and a sweetener.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid compositions. Examples of such carriers include magnesium stearate, starch, lactose, sucrose, microcrystalline cellulose and binders, for example polyvinylpyrrolidone. The tablet can also be provided with a color film coating, or color included as part of the carrier(s). In addition, active compound can be formulated in a controlled release dosage form as a tablet comprising a hydrophilic or hydrophobic matrix.

A composition in the form of a capsule can be prepared using routine encapsulation procedures, for example by incorporation of active compound and excipients into a hard gelatin capsule. Alternatively, a semi-solid matrix of active compound and high molecular weight polyethylene glycol can be prepared and filled into a hard gelatin capsule; or a solution of active compound in polyethylene glycol or a suspension in edible oil, for example liquid paraffin or fractionated coconut oil can be prepared and filled into a soft gelatin capsule. Compound of Formula 1 and their pharmaceutically acceptable salts which are active when given parenterally can be formulated for intramuscular or intravenous administration.

A typical composition for intra-muscular administration will consist of a suspension or solution of active ingredient in an oil, for example arachis oil or sesame oil. A typical composition for intravenous administration will consist of a sterile isotonic aqueous solution containing, for example active ingredient, dextrose, sodium chloride, a co-solvent, for example polyethylene glycol and, optionally, a chelating agent, for example ethylenediamine tetracetic acid and an anti-oxidant, for example, sodium metabisulphite. Alternatively, the

solution can be freeze dried and then reconstituted with a suitable solvent just prior to administration.

Compounds of structure (1) and their pharmaceutically acceptable salts which are active on rectal administration can be formulated as suppositories. A typical suppository formulation will generally consist of active ingredient with a binding and/or lubricating agent such as a gelatin or cocoa butter or other low melting vegetable or synthetic wax or fat.

Compounds of Formula 1 and their pharmaceutically acceptable salts which are active on topical administration can be formulated as transdermal compositions. Such compositions include, for example, a backing, active compound reservoir, a control membrane, liner and contact adhesive.

The typical daily dose of a compound of Formula 1 varies according to individual needs, the condition to be treated and with the route of administration. Suitable doses are in the general range of from 0.001 to 10 mg/kg bodyweight of the recipient per day.

Within this general dosage range, doses can be chosen at which the compounds of Formula 1 lower plasma cholesterol levels and raise metabolic rate with little or no direct effect on the heart. In general, but not exclusively, such doses will be in the range of from lower doese (0.001 to 0.5 mg/kg) to higher doses (0.5 to 10 mg/kg).

In addition, within the general dose range, doses can be chosen at which the compounds of Formula 1 lower plasma cholesterol levels and have little or no effect on the heart without raising metabolic rate. In general, but not exclusively, such doses will be in the range of from 0.001 to 0.5 mg/kg.

It is to be understood that the 2 sub ranges noted above are not mutually exclusive and that the particular activity encountered at a particular dose will depend on the nature of the compound of Formula 1 used.

Preferably, the compound of Formula 1 is in unit dosage form, for example, a tablet or a capsule so that the patient may self-administer a single dose. In general, unit doses contain in the range of from 0.05-100 mg of a compound of Formula 1. Preferred unit doses contain from 0.05 to 10 mg of a compound of Formula 1.

The active ingredient may be administered from 1 to 6 times a day. Thus daily doses are in general in the range of from 0.05 to 600 mg per day. Preferably, daily doses are in the range of from 0.05 to 100 mg per day. Most preferably from 0.05 to 5 mg per day.

EXAMPLES

EXAMPLE 1 - SYNTHESIS OF TR LIGANDS

Many TR ligands are known in the art, including T4 (thyroxine), T3, T2 and TS-9. See Jorgensen, Thyroid Hormones and Analogs, in 6 *Hormonal Proteins and Peptides, Thyroid Hormones* 107-204 (Choh Hao Li ed., 1978), incorporated by reference herein.

The syntheses of several TR ligands are described below.

Synthesis of TS1, TS2, TS3, TS4, TS5

TS1, TS2, TS3, TS4 and TS5 and analogs thereof can all be prepared by simple acylation of the nitrogen atom of any thyronine analog, including T3 (3,5,3'-triiodo-L-thyronine), T4 (thyroxine) and 3,5-diiodothyronine. TS1 and TS2 are synthesized by reacting T3 with Ph_2CHCO_2NHS (N-hydroxy succinimide-2,2-diphenylacetate) and $C_{16}H_{33}CO_2NHS$, respectively. TS3 is synthesized by reacting T3 with FMOC-Cl (fluorenylmethyloxycarbonylchloride). TS4 is synthesized by reacting T3 with $tBOC_2O$ (tBOC anhydride or di-t-butyldicarbonate). TS5, which differs from TS1-4 by having a -H instead of an -I at the R^1_3 position, is synthesized by reacting 3,5-diiodothyronine with $tBOC_2O$. The general reaction scheme for TS1, TS2, TS3, TS4 and TS5 is depicted in **FIG. 11.** It should be noted that in the reaction scheme, both TS5 and its precursor both have a hydrogen rather than an iodine at the R^1_3 position.

Synthesis of TS6 and TS7

TS6 is synthesized by reacting TS5 with paranitrophenylisocyanate. TS7 is synthesized by reacting TS6 with TFA (trifluoroacetic acid), which cleaves the tBOC group. These reactions are simple organic synthesis reactions that can be performed by anyone of ordinary skill in the art. The synthetic scheme for TS6 and TS7 is diagrammed in **FIG. 12.**

Synthesis of TS8

TS8 is synthesized by reacting TS5 with Ph_2CHNH_2 (diphenylmethylamine) in the presence of triethylamine and any amide forming condensing reagent, such as TBTU (hydroxybenztriazoleuronium tetrafluoroborate) or HBTU (hydroxybenztriazoleuronium hexafluorophosphate). The synthesis scheme for TS8 is depicted in **FIG. 13.**

SYNTHESIS OF 3,5-DIIODO-3'ISOPROPYLTHYRONINE DERIVATIVES

For designing a class of antagonists, it is important to have a hydrophobic group at the 3' position as well as an extension at the 5' position. Preferred hydrophobic groups at the 3' position include: methyl, benzyl, phenyl, iodo, and heterocyclic structures. The synthesis of a 3,5-diiodo-3'-isopropyl-5'-substituted thyronine is described below. The example provided describes the specific steps for synthesizing the TS10 compound, but this general reaction scheme can be used by one of ordinary skill in the art to synthesize any number of 3,5,-diiodo-3'-isopropyl-5'-substituted thyronine derivatives, which are characterized by having an extension at the 5' position. Additional compounds of this class can be synthesized using known organic synthesis techniques.

The synthesis of TS10 is described below and is depicted in **FIG. 14.** Numbers used in the reaction scheme for TS10 indicating the reaction product for each step are in parentheses.

2-Formyl-6-isopropylanisole (1): 2-formyl-6-isopropylanisole (10.0 g, 61 mmol), as-made by Casiraghi, *et al.* JCS Perkin I, 1862 (1980) (incorporated by reference), is added dropwise to a suspension of sodium hydride (3.7 g, 153 mmol) in 50 mL THF and 50 mL of DMF in a round bottom flask. The addition generates an exothermic reaction and formation of a gray solid. Methyl iodide (26.0 g, 183 mmol) is then added dropwise and the reaction mixture is stirred at room temperature for 5 hours. The reaction mixture is quenched with 20 mL of water, then poured into 500 mL of water, and is extracted with ether (2 x 300 mL). The ether layers are combined, washed with water (5 x 1000 mL), dried over magnesium sulfate and concentrated in vacuo to provide 10.2 g (94%) of the title compound, with the following ^{1}H NMR ($CDCl_3$) properties: d 10.30 (s, 1H), 7.63 (d, 1H, J=3 Hz), 7.50 (d, 1H, J=3 Hz), 7.13 (t, 1H, J=3 Hz), 3.81 (s,'3H), 3.31 (heptet, 1H, J=7.5 Hz), 1.19 (d, 6H, J=7.5 Hz).

2-(2-Hydroxynonyl)-6-isopropylanisole (not shown in scheme): Octylmagnesium chloride (8.4 mL, 16.9 mmol, 2.0 M) is added dropwise to a solution of 1 (1.5 g, 8.4 mmol) in 10 mL THF at -78°C. The reaction mixture is stirred for 2 hours with warming to room temperature. The reaction mixture is diluted with 50 mL ether and poured into 50 mL water. The ether layer is washed with brine (1 x 50 mL), dried over sodium sulfate, and

concentrated in vacuo. Flash chromatography (silica gel, 10% ether/hexane → 15% ether/hexane) provides 734 mg (30%) of the title compound with the following ^{1}H NMR ($CDCl_3$) properties: d 7.33-7.10 (m, 3H), 5.00 (br. s, 1H), 3.81 (s, 3H), 3.33 (heptet, 1H, J=7 Hz) 1.90-1.19 (m, 14H), 0.86 (t, 3H, J=6.5 Hz); HRMS (EI), found: 292.2404; calc'd: 292.2402.

2-nonyl-6-isopropylanisole (2): Compound **2** (663 mg, 2.3 mmol) is dissolved in solution of 5 mL ethanol and 5 mL acetic acid, and a spatula tip of palladium on carbon catalyst is added. The reaction mixture is then charged with hydrogen gas (using a simple balloon and needle) and the mixture is stirred at room temperature overnight. The next day, the reaction mixture is poured into ether (100 mL) and the ether layer is extracted with saturated sodium bicarbonate (3 x 100 mL). The ether layer is dried over sodium sulfate and concentrated *in vacuo* to provide 581 mg (91%) of **(2)** with the following ^{1}H NMR ($CDCl_3$) properties: d 7.14-7.00 (m, 3H), 3.75 (s, 3H), 3.36 (heptet, 1H, J=6.8 Hz), 2.63 (t, 2H, J=7.5 Hz), 1.68-1.15 (m, 14H), 0.86 (t, 3H, J=5.5 Hz); HRMS (EI), mass found: 276.2459; calculated: 276.2453.

Thyronine adduct (4): Fuming nitric acid (0.071 mL) is added to 0.184 mL acetic anhydride chilled to -5°C. Iodine (66 mg) is added to this mixture followed by trifluoroacetic acid (0.124 mL). This mixture is stirred for 1 hour with warming to room temperature, at which point all of the iodine is dissolved. The reaction mixture was then concentrated *in vacuo* to provide an oily semi-solid material. The residue was dissolved in 0.7 mL of acetic anhydride and cooled to -20°C. A solution of anisole **(2)** (581 mg, 2.1 mmol) in 1.2 mL acetic anhydride and 0.58 mL TFA is added dropwise. The reaction mixture is stirred at -20° for 1 hour, then stirred overnight with warming to room temperature. The reaction mixture is partitioned between water and methylene chloride. The methylene chloride layer is dried over sodium sulfate and concentrated *in vacuo* to provide the iodonium salt **(3)** as an oil. This material is not purified or characterized, and is directly introduced into the coupling reaction.

N-Trifluoroacetyl-3,5-diiodotyrosine methyl ester (552 mg, 1.0 mmol) prepared according to the procedure of N. Lewis and P. Wallbank, *Synthesis* 1103 (1987) (incorporated by reference) and all of the crude iodonium salt **(3)** from above is dissolved in 5 mL of anhydrous methanol. Diazabicyclo**[5.4.0]**undecane (DBU) (183 mg, 1.2 mmol) and

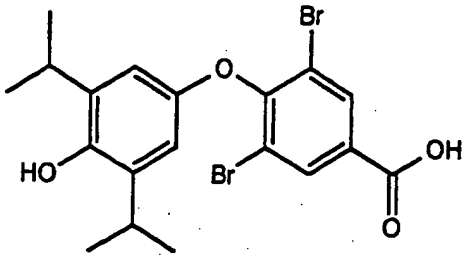
a spatula tip of copper-bronze are added and the resulting mixture is stirred at room temperature overnight. The next day, the reaction mixture is filtered, and the filtrate is concentrated *in vacuo*. The crude residue is purified by flash chromatography (silica gel, 10% ethyl acetate/hexane) to provide 30 mg (4%) of the protected thyronine adduct (**4**).

Deprotected thyronine (TS10): The protected thyronine **4** (30 mg, 0.04 mmol) is dissolved in a mixture of 2.25 mL acetic acid and 2.25 mL 49% hydrobromic acid. The reaction mixture is heated to reflux for 5 hours. The reaction mixture is cooled to room temperature, and the solvents are removed *in vacuo*. Water is added to triturate the oily residue into a gray solid. This solid material is filtered, washed with water, and dried over P_2O_5 *in vacuo* to provide 24 mg (81%) of the title compound, TS10, with the following ^1H NMR ($CDCl_3$) properties: d 7.57 (s, 1H), 6.86 (s, 1H), 6.45 (s, 1H), 6.34 (s, 1H), 4.81 (m, 1H), 3.86 (s, 3H), 3.71 (s, 3H), 3.33-3.05 (m, 3H), 2.58-2.47 (m, 2H), 1.62-0.76 (m, 23H); MS (LSIMS): $M^+ = 817.0$.

As mentioned above, this reaction scheme can be modified by one of ordinary skill in the art to synthesize a class of compounds characterized by 3,5-diiodo-3'isopropylthyronine derivatives, wherein (1) the 3' isopropyl group can be replaced with a hydrophobic group, including methyl, benzyl, phenyl, iodo, and heterocyclic structures, and (2) a wide variety of chemical structures can be incorporated at the 5' position, including alkyl groups, planar aryl, heterocyclic groups, or polar and/or charged groups.

The aldehyde (**1**) in the above reaction scheme is a versatile synthetic intermediate which allows for the attachment of a variety of chemical moieties to the 5' position of the final thyronine derivative. In addition, a variety of chemical reactions can be used to attach the chemical moieties. These reactions are well known in the art and include organometallic additions to the aldehyde (including Grignard reagents,'organolithiums, etc.), reductive amination reactions of the aldehyde with a primary or secondary amine, and Wittig olefination reactions with a phosphorous ylid or stabilized phosphonate anion. Other possibilities include reduction of the aldehyde to a benzyl alcohol allowing for etherification reactions at the 5' position. As mentioned above, these methods allow for a wide variety of chemical structures to be incorporated at the 5' position of the final thyronine derivative, including alkyl groups, planar aryl, heterocyclic groups or polar and/or charged groups.

Synthesis of 3, 5-dibromo-4-(3',5'-diisopropyl-4'-hydroxyphenoxy) benzoic acid (Compound 11).



(a) A mixture of 2,6-diisopropyl phenol (20 g, 0.11 mol), potassium carbonate (62 g, 0.45 mol), acetone (160 ml) and methyl iodide (28 ml, 0.45 mole) is refluxed for three days. The reaction mixture is filtered through celite, evaporated, dissolved in ether, washed twice with 1M sodium hydroxide, dried over magnesium sulphate and concentrated to afford 15.1 g (0.08 mol, 70%) of 2,6-diisopropyl anisole as a slightly yellow oil.

(b) Fuming nitric acid (12.4 ml, 265 mmol) is added dropwise to 31.4 ml of acetic anhydride which is cooled in a dry ice/carbon tetrachloride bath. Iodine 11.3 g, 44.4 mmol) is added in one portion followed by dropwise addition of trifluoroacetic acid (20.5 ml, 266 mmole). The reaction mixture is stirred at room temperature until all the iodine is dissolved. Nitrogen oxides are removed by flushing nitrogen into the vessel. The reaction mixture is concentrated, the residue is dissolved in 126 ml of acetic anhydride and is cooled in a dry ice/carbon tetrachloride bath. To the stirred solution 2,6-diisopropylanisole (51 g, 266 mmol) in 150 ml of acetic anhydride and 22.6 ml of trifluoroacetic acid is added dropwise. The reaction mixture is left to stand at room temperature over night and then is concentrated. The residue is taken up in 150 ml of methanol and treated with 150 ml of 10% aqueous sodium bisulfite solution and 1 liter of 2M sodium borotetrafluoride solution. After the precipitate aggregates, petroleum ether is added and the supernatant is decanted. The precipitate is triturated with petroleum ether, filtered, washed with petroleum ether and dried at room temperature in vacuo. This affords 34 g (57 mmol, 65%) of bis(3,5-diisopropyl-4-methoxyphenyl)iodonium tetrafluoroborate as a white solid.

(c) To a stirred solution of 3,5-dibromo-4-hydroxybenzoic acid (12 g, 40.5 mmol) in 250 ml of methanol, thionyl chloride (3 ml) is added dropwise. The reaction mixture is refluxed for five days, water is added and the precipitated product is filtered off. The residue is dissolved in ethyl acetate. From the aqueous phase, methanol is removed by

concentration. The aqueous phase is then saturated with sodium chloride, and extracted with ethyl acetate. The combined organic phases are dried over magnesium sulphate, filtered and concentrated. This gives 12.5 g (40.5 mmol, 100%) of 3,5-dibromo-4-hydroxymethyl benzoate as a white crystalline solid.

(d) The products obtained in steps b and c are reacted with each other according to the following protocol. To bis(3,5-diisopropyl-4-methoxyphenyl)iodonium tetrafluoroborate (2.86 g, 4.8 mmole) and copper bronze (0.42 g, 6.4 mmole) in 7 ml. of dichloromethane at 0°C is added dropwise a solution of 3,5-dibromo-4-hydroxymethyl benzoate (1.0 g, 3.2 mmole) and triethylamine (0.36 g, 3.5 mmole) in 5 ml of dichloromethane. The reaction mixture is stirred in the dark for eight days and then is filtered through celite. The filtrate is concentrated and the residue is purified by column chromatography (silica gel, 97:3 petroleum ether/ethyl acetate) to give 0.62 g (1.2 mmole, 39%) of 3,5-dibromo-4-(3',5'-diisopropyl-4'-methoxyphenoxy)methyl benzoate as a solid.

(e) The product from step d (0.2 g, 0.4 mmole) is dissolved in 2 ml. dichloromethane, is put under nitrogen and is cooled at -40°C. To the stirred solution is added 1M BBr_3 (1.2 ml, 1.2 mmole) dropwise. The reaction mixture is allowed to reach room temperature and then is left over night. It is cooled to 0°C and then hydrolyzed with water. Dichloromethane is removed by concentration and the aqueous phase is extracted with ethyl acetate. The organic phase is washed with 1M hydrochloric acid and brine. Then it is dried over magnesium sulphate, filtered and concentrated. The residue is chromatographed (silica, 96:3.6:0.4 dichloromethane/methanol/acetic acid) producing 93 mg (0.2 mmole, 51%) of 3,5-dibromo-4-(3',5'-diisopropyl-4'-hydroxyphenoxy)benzoic acid as a white solid. ^1H nmr ($CDCl_3$) δ 1.23 (d, 12H, methyl), 3.11 (m, 2H, CH), 6.50 (s, 2H, 2,6-H) 8.33 (s, 2H, 2',6'-H).

Synthesis of addition ligands are described in U.S. Serial No. 08/877,792, filed June 18, 1997 which is herein incorporated in its entirety by reference.

TABLE 2 and **FIG. 15** depict the structures of several TR ligands in reference to Formula I.

TABLE 2

Cmpd	R_3	R_4	R_5	R'_3	R'_4	R'_5	R_1
*T_3	-I	-O-	-I	-I	-OH	-H	$-CH_2CH(NH_2)CO_2H$
*T_4	-I	-O-	-I	-I	-OH	-I	$-CH_2CH(NH_2)CO_2H$
TS1	-I	-O-	-I	-I	-OH	-H	$-CH_2CH[NHCOCH\phi_2]CO_2H$
TS2	-I	-O-	-I	-I	-OH	-H	$-CH_2CH[NHCO(CH_2)_{15}CH_3]CO_2H$
TS3	-I	-O-	-I	-I	-OH	-H	$-CH_2CH[NH\text{-}FMOC]CO_2H$
TS4	-I	-O-	-I	-I	-OH	-H	$-CH_2CH[NH\text{-}tBOC]CO_2H$
TS5	-I	-O-	-I	-H	-OH	-H	$-CH_2CH[NH\text{-}tBOC]CO_2H$
TS6	-I	-O-	-I	-H	$-OC(O)NH=\emptyset_pNO_2$	-H	$-CH_2CH[NH\text{-}tBOC]CO_2H$
TS7	-I	-O-	-I	-I	$-OC(O)NH=NH\emptyset NO_2$	-H	$-CH_2CH(NH_2)CO_2H$
TS8	-I	-O-	-I	-H	$-NH\text{-}CH\emptyset\emptyset$	-H	$-CH_2CH[NH\text{-}tBOC]CO_2H$
TS9	-I	-O-	-I	-IsoPr	-OH	-H	$-CH_2CH(NH_2)CO_2H$
TS10	-I	-O-	-I	-IsoPr	-OH	$-(CH)_8-CH_3$	$-CH_2CH(NH_2)CO_2H$

* Prior Art Compound

-Ø: phenyl

$-\emptyset pNO_2$: para nitro phenyl

EXAMPLE 2 - RECEPTOR BINDING ASSAYS OF TR LIGANDS

To test the ability of synthesized TR ligands to bind to a thyroid receptor (TR), the binding affinity of a TR ligand for TR is assayed using TR's prepared from rat liver nuclei and 125_I T_3 as described in J.D. Apriletti, J.B. Baxter, and T.N. Lavin, *J. Biol. Chem.*, *263*: 9409-9417 (1988). The apparent Kd's are calculated using the method described by Apriletti (1995) and Apriletti (1988). The apparent Kd's are presented in **TABLE 3**. The apparent Kd's (App.Kd) are determined in the presence of the sample to be assayed, 1 nM $[^{125}I]T_3$, and 50μg/ml core histones, in buffer E (400 mM KCl, 200 mM potassium phosphate, pH 8.0, 0.5 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT) in a volume of 0.21 ml.

After incubation overnight at 4°C, 0.2 ml of the incubation mixture is loaded onto a Quick-Sep Sephadex G-25 column (2.7 x 0.9 cm, 1.7 ml bed volume) equilibrated with buffer E. The excluded peak of protein-bound [^{125}I]T_3 is eluted with 1 ml of buffer E, collected in a test tube, and counted. Specific T_3 binding is calculated by subtracting nonspecific binding from total binding.

TABLE 3

Compound	App.Kd(nM)	Coactivation Assay RIP-140	EC_{50}(M)
T_3	0.06	+	10^{-10}
T_4	2	+	10^{-9}
TS1	4	+	10^{-7}
TS2	1400	nd	nd
TS3	4	+	10^{-8}
TS4	8	+	nd
TS5	220	+	10^{-6}
TS6	>10000	nd	nd
TS7	260	+	10^{-7}
TS8	6000	nd	nd
TS9	1	+	10^{-10}
TS10	400	+	10^{-6}

+: RIP-140 Binding

-: RIP-140 Binding

nd: Not Determined

EXAMPLE 3 - INCREASED NUCLEAR PROTEIN COACTIVATION BY TR LIGANDS

To test the ability of TR ligands to activate the binding of TR to the nuclear activation protein RIP-140 (a nuclear protein that can bind to nuclear receptors, such as the estrogen receptor), a TR ligand is liganded to TR and then incubated with RIP-140 as described in V. Cavailles, *et al.*, *EMBO J.*, 14(15):3741- 3751 (1995), which is incorporated by reference herein. In this assay, 35_S-RIP-140 protein binds to liganded TR but not unliganded TR. Many TR 35_S ligands can activate RIP-140 binding as shown in **TABLE 3.**

EXAMPLE 4 - TR LIGAND BINDING AND TR ACTIVATION IN CULTURED CELLS

To test TR activation of transcription in a cellular environment, TR ligands are assayed for their ability to activate a reporter gene, chloramphenicol transferase ("CAT"), which has a TR DNA binding sequence operatively linked to it. Either GC or L937 cells (available from the ATCC) can be used, respectively). In such assays, a TR ligand crosses the cell membrane, binds to the TR, and activates the TR, which in turn activates gene transcription of the CAT by binding the TR DNA binding region upstream of the CAT gene. The effective concentration for half maximal gene activation (EC_{50}) is determined by assaying CAT gene activation at various concentrations as described herein and in the literature. The results of CAT gene activation experiments are shown in **TABLE 3.**

CAT GENE ACTIVATION ASSAYS

Functional response to thyroid hormone (3,5,3'-triiodo-L-thyronine, T_3) and TR ligands is assessed either in a rat pituitary cell line, GC cells, that contain endogenous thyroid hormone receptors (TRs) or U937 cells that contain exogenous TRs expressed as known in the art. GC cells are grown in 10-cm dishes in RPMI 1640 with 10% newborn bovine serum, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. For transfections, cells are trypsinized, resuspended in buffer (PBS, 0.1% glucose) and mixed with a TREtkCAT plasmid (10 mg) or phage in 0.5 ml buffer (15$\pm$5 million cells) and electroporated using a Bio-Rad gene pulser at 0.33 kvolts and 960 mF. The TREtkCAT plasmid contains two copies of a T_3 response element (AGGTCAcaggAGGTCA) cloned in the Hind III site of the pUC19 polylinker immediately upstream of a minimal (-32/+45) thymidine kinase promoter linked to CAT (tkCAT) coding sequences. After electroporation,

cells are pooled in growth medium (RPMI with 10% charcoal-treated, hormone stripped, newborn bovine serum), plated in 6-well dishes and treated with either ethanol or hormone. CAT activity is determined 24 hours later as described D. C. Leitman, R. C. J. Ribeiro, E. R. Mackow, J. D. Baxter, B. L. West, *J. Biol. Chem.* 266, 9343 (1991), which is incorporated by reference herein.

EFFECT OF TS-10 ON THE TRANSCRIPTIONAL REGULATION OF THE DR4-ALP REPORTER GENE IN THE PRESENCE OR ABSENCE OF T3.

Characteristics of the TRAF cells: TRAFa1 are CHO K1 cells stably transformed with an expression vector encoding the human thyroid hormone receptor α 1 and a DR4,ALP reporter vector; TRAFb1 are CHO K1 cells stably transformed with an expression vector encoding the human thyroid hormone receptor β1 and a DR4-ALP reporter vector.

Interpretation of the effect of compound TS-10 on the transcriptional regulation of the DR4-ALP reporter gene in the presence or absence of T3.

TRAFa1 reporter cells: TS-10 alone (open circles) induces a partial activation of the expression of the ALP reporter protein amounting to approximately 27% of the maximal effect by the natural thyroid hormone T3. In the presence of T3 (filled circles), TS-10 has a weak antagonistic effect. The EC50 concentration for the agonistic effect of TS-10 and the EC50 concentration for its T3 antagonistic effect, respectively, is indicated in **FIG. 18**.

In **FIG. 18**, open and filled circles with dotted lines show the dose-dependent effect of TS-10/T3 on the toxicity marker (MTS/PMS), reduction of tetrazolium salt in the mitochondria, displayed on the right y-axis as optical density. There is no obvious toxic effect of TS-10 on the MTS-PMS marker but there is a clear effect on the morphology of the cells, as can be seen under the light microscope, at the highest concentration of TS-10 (32 mM) both in the absence and presence of T3, respectively (not shown in the figure).

TRAFb1 reporter cells: TS-10 alone (open circles) induces a partial activation of the expression of the ALP reporter protein amounting to approximately 35% of the maximal effect by T3. The EC50 concentration for the agonistic effect of TS-10 is indicated in **FIG. 19.** In the presence of T3 (filled circles), TS-10 shows, if anything, a slight potentiation of the T3 effect on the expression of the ALP reporter protein. The T3 inhibitory effect of TS-10 at its highest concentration used (32 mM) is a toxic effect rather than T3 antagonism.

In **FIG. 19,** open and filled circles with dotted lines show the dose-dependent effect of TS-10/T3 on the toxicity marker (MTS/PMS), reduction of tetrazolium salt in the mitochondria, displayed on the right y-axis as optical density. There is no obvious toxic effect of TS-10 on the MTS-PMS marker but a clear effect on the morphology of the cells can be observed, under the light microscope, at the highest concentration of TS-10 (32 mM) both in the absence and presence of T3, respectively (not shown in the figure).

HepG2 (HAF18) reporter cells: TS-10 alone (open circles) induces a partial activation of the expression of the ALP reporter protein amounting to slightly more than 50% of the maximal effect by T3. The EC50 concentration for the agonistic effect of TS-10 is indicated in **FIG. 20.** In the presence of T3 (filled circles), TS-10 shows no effect i.e. no T3 antagonism nor potentiation/additive effect to T3. Open and filled circles with dotted lines show the dose-dependent effect of TS-10/T3 on the toxicity marker (MTS/PMS), reduction of tetrazolium salt in the mitochondria, displayed on the right y-axis as optical density. There is no obvious toxic effect of TS-10 on the MTS/PMS marker or on the morphology of the cells, as can be observed using a light microscope, at any concentration of TS-10/T3 used.

Example 5 - Comparisons of Human TR-α and Human TR-β

Competition for [^{125}I]T_3 binding to TR LBD by T_3 and Triac

The drug, Triac, is a thyroid hormone agonist. Triac is 3,5,3'-triiodothyroacetic acid and is described in Jorgensen, Thyroid Hormones and Analogs in 6 *Hormonal Proteins and Peptides, Thyroid Hormones* at 150-151 (1978). Another compound that can be used in place of Triac is 3,5-diiodo-3'-isopropylthyroacetic acid. Competition assays are performed to

compare the displacement of [^{125}I]T_3 from binding with human TR-α LBD or human TR-β LBD by unlabeled T_3 or Triac. The results of such assays are depicted in **FIG. 16.**

Standard binding reactions are prepared containing 1 nM [^{125}I]T_3, 30 fmol of human TR-α (empty symbols) or β (solid symbols), and various concentrations of competing unlabeled T_3 (circles) or Triac (triangles). Assays are performed in duplicate.

Competition for [^{125}I]T_3 binding to variant TR LBD by T_3, Triac and GC-1

The following assays residues involved in selective binding among TR isoforms. Competition assays are performed to compare the displacement of [^{125}I]T_3 from binding with wild-type human TR-α LBD or human TR-β LBD, to a variant form of the TR LBDs by unlabeled T_3, Triac or GC-1. A variant TR-α or TR-β is constructed by substituting an amino acid found in the corresponding position of the other TR isoform. For example, asparagine 331 in human TR-β corresponds to serine 277 in human TR-α. To test binding specificity contributed by this position, a variant human TR-β is constructed that contains asparagine 331 substituted with a serine residue (designated Asn331Ser or N331S). Binding assays are described in *Apriletti et al.* (Protein Expression and Purification 6:363-370 (1995)). The results of such assays are depicted in FIG. 27, and summarized in Table 4 below.

TABLE 4

Effect of TR-β Substitution N331S on Binding Affinity

Ligand	Native TR-α	Native TR-β	Mutant TR-β
T3	20 pM	60 pM	100 pM
T4	600	3000	ND
Triac	20	20	100
$IpBr_2$	17	ND	ND
Dimit	6000	8000	ND
GC-1	200	40	400

Competition curves comparing wildtype TR-β versus the variant TR-β N331S for binding T3, Triac or GC-1 show that the affinity of the mutant receptor for Triac was reduced to approximately the same as for T3 (vs. 3-fold greater in wild type) so that the relative affinities are similar to wild-type TR-α. The affinity for GC-1 was also reduced to several fold less than T3, as is seen with TR-α.

Comparison of the affinity of TR-β variant N331S to the native TRs for selected ligands is as follows:

Native TR-α for various ligands (T3, T4, Triac, IpBr2, Dimit, GC-1):

$IpBr_2$ > Triac ≃ T3 > GC-1 > T4 > Dimit

Native TR-β (T3, T4, Triac, Dimit, GC-1)

Triac > GC-1 ≥ T3 > T4 > Dimit

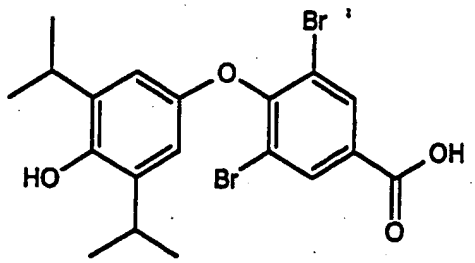
Variant TR-β (N331S) (T3, Triac, GC-1)

Triac ≃ T3 > GC-1.

Scatchard Analysis of [^{125}I]T_3 Binding to TR

Human TR-α (left panel) or human TR-β (right panel) is assayed for T_3 binding in the presence of increasing concentrations of [^{125}I]T_3. The apparent equilibrium dissociation constant (20 pM for α and 67 pM for β) is calculated by linear regression analysis and is depicted in **FIG. 17.**

3, 5-DIBROMO-4-(3',5'-DIISOPROPYL-4'-HYDROXYPHENOXY) BENZOIC ACID IS A TR-α SELECTIVE SYNTHETIC LIGAND.



3, 5-dibromo-4-(3',5'-diisopropyl-4'-hydroxyphenoxy) benzoic acid (Compound 11), the structure of which is drawn above, is assayed for binding to the two different isoforms of the TR, TR-α and TR-β. Compound 11 exhibits an IC50 of 1.6 μM for binding to TR-α

and an IC50 of 0.91 μM for binding to TR-β. Assays for determining selective binding to the TR-α or TR-β LBD can include reporter assays, as described herein. See also Hollenberg, *et al.*, *J. Biol. Chem.*, (1995) *270*(24):14274-14280.

EXAMPLE 6 - PREPARATION AND PURIFICATION OF A TR-α LBD

Rat TR-α LBD, residues Met122 - Val410, is purified from *E. coli* ("LBD-122/410"). The expression vector encoding the rat TR-α LBD is freshly transfected into *E. coli* strain BL21(DE3) and grown at 22°C in a 50-liter fermenter using 2x LB medium. At an A_{600} of 2.5-3, IPTG is added to 0.5 mM and growth is continued for 3 h before harvesting. The bacterial pellet is quickly frozen in liquid nitrogen and stored at -70°C until processed. Extraction and purification steps are carried out at 4°C. The bacteria are thawed in extraction buffer (20MM Hepes, pH 8.-, 1 mM EDTA, 0.1% MTG, 0.1 mM PMSF, and 10% glycerol) at a ratio of 10 ml buffer/g bacteria. Bacteria are lysed by incubation for 15 min. with 0.2 mg/ml lysozyme and sonicated at maximum power while simultaneously homogenized with a Brinkmann homogenizer (Model PT 10/35 with generator PTA 35/2) until the solution loses its viscosity. After centrifugation for 10 min at 10,000 g, the supernatant is adjusted to 0.4 M KCl, treated with 0.6% PEI to precipitate fragmented DNA, and centrifuged for 10 min at 10,000 g. The rat TR-α LBD in the supernatant is then precipitated with 50% ammonium sulfate and centrifuged for 10 min at 10,000 g. The precipitate is resuspended with buffer B (20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.01% Lubrol, and 10% glycerol) to a final conductivity of 9 mS/cm (approx. 0.7 M ammonium sulfate) and centrifuged 1 h at 100,000g. The supernatant is frozen in liquid nitrogen and stored at -70°C.

The crude extract is thawed, bound with a tracer amount of [^{125}I]T_3, and loaded directly onto a phenyl-Toyopearl hydrophobic interaction column (2.6 x 18 cm, 95 ml bed volume) at 1.5 ml/min. The column is eluted with a 2-h gradient from 0.7 ammonium sulfate, no glycerol to no salt, 20% glycerol in buffer C (20 mM Hepes, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF). The rat TR-α LBD prebound to tracer [^{125}I]T_3 (less than 0.005% of total rat TR-α LBD) is detected using a flow-through gamma emission detector, whereas unliganded rat TR-α LBD is assayed by postcolumn [^{125}I]T_3 binding assays (described herein).

The phenyl-Toyopearl unliganded rat TR-α LBD peak fractions are pooled, diluted with buffer B to a conductivity of 0.5 mS/cm (equivalent to approx. 20 mM ammonium sulfate), loaded onto a TSK-DEAE anion-exchange column (2 x 15 cm, 47 ml bed volume) at 4 ml/min, and eluted with a 60-min gradient from 50 to 200 mM NaCl in buffer B.

The unliganded rat TR-α LBD peak fractions from TSK-DEAE are pooled, diluted twofold with buffer B, loaded at 0.75 ml/min on a TSK-heparin HPLC column (0.8 x 7.5 cm, 3 ml bed volume), and eluted with a 50 to 400 mM NaCl gradient in buffer B.

The pool of unliganded rat TR-α LBD peak fractions from the TSK-heparin column is adjusted to 0.7 M ammonium sulfate, loaded at 0.75 ml/min on a TSK-phenyl HPLC column (0.8 x 7.5 cm, 3 ml bed volume), and eluted with a 60-min gradient from 0.7 M ammonium sulfate without glycerol to no salt with 20% glycerol in buffer C. The fractions containing unliganded rat TR-α LBD are pooled and incubated with a five fold excess of hormone for 1 h, the salt concentration is adjusted to 0.7 M ammonium sulfate, and the sample is reloaded and chromatographed on the same column as described above.

EXAMPLE 7 - CRYSTALLIZATION OF LIGANDED TR-α LBD

Material from a single LBD-122/410 preparation is divided into batches, and quantitatively bound with one of the following ligands: Dimit, T_3, or Triac $IpBr_2$ (3,5dibromo-3'isopropylthyronine) for the final purification step.

To maintain full saturation of rat TR-α LBD with a ligand, and to prepare the complex for crystallization, the ligand-bound rat TR-α LBD is concentrated and desalted in an Amicon Centricon-10 microconcentrator (McGrath et al, *Biotechniques*, (1989) *7*:246-247, incorporated by reference herein), using 10 mM Hepes (pH 7.0), 3.0 mM DTT, and 1.0 nM to 10 nM ligand.

Factorial crystallization screening trials (Jancarik & Kim, *J. Appl. Crystallogr.* (1991) *24*:409-411, incorporated by reference herein) are carried out for rat TR-α LBD bound to selected ligands using hanging-drop vapor diffusion at 17°C (with 1 μl protein solution, 1 μl precipitant solution and a 0.5 ml reservoir using silanized coverslip: (McPherson, Preparation and Analysis of Protein Crystals (1982), incorporated by reference herein). Rat TR-α LBD is not stable at 4°C and is stored at -80°C, where it maintains its avidity for hormone and its crystallizability for approximately two to three months. These procedures are carried out as

described in McGrath, M.E. *et al.*, *J. Mol. Biol.* (1994) *237*:236-239 (incorporated by reference). Crystals are obtained in condition 21 of the screening trials (Jancarik & Kim 1991) and conditions are then optimized. Wedge-shaped crystals are reproducibly obtained with hanging-drop vapor fusion at 22°C with 15% 2-methyl-2,4-pentanediol (MPD), 0.2 M ammonium acetate and 0.1 M sodium cacodylate (pH 6.7), 3 mM DTT, with 2 μl protein solution, 1 μl precipitant solution and a 0.6 ml reservoir using silanized coverslip, and with 8.7 mg/ml (Dimit), 5.5 mg/ml ($IpBr_2$), 5 mg/ml (Triac), or 2.3 mg/ml (T_3) over a period of three days. Under these conditions, diffraction quality crystals (dimension 0.5 x 0.2 x 0.0075 mm^3) can be grown at ambient temperature (22°C). The best crystals have a limiting dimension of approximately 100 μm and are obtained at a protein concentration between 2.3 and 8.7 mg/ml in the presence of 3 mM DTT. The crystals are of the monoclinic space group C2, with one monomer in the asymmetric unit.

EXAMPLE 8 - CRYSTALLIZATION OF HUMAN TR-β LBD COMPLEXED WITH T3, TRIAC, OR GC-1

Human TR-β LBD complexed with T_3, Triac, or GC-1 are purified according to the same procedures described above for the rat TR-α LBD, with the following modifications.

The expression of human TR-β LBD differs from the rat TR-α LBD in that the human TR-β LBD residues extend from the amino acid at position 716 through the amino acid at position 1022, according to the amino acid numbering scheme for the various nuclear receptor LBDs depicted in **FIG. 3**. **FIG. 3** illustrates a numbering scheme applicable to all of the nuclear receptors listed as well as to any additional homologous nuclear receptors. The vertical lines on **FIG. 3** at position 725 and at position 1025 delineate the preferred minimum amino acid sequence necessary to obtain adequate binding of ligand. The amino acid sequence from position 716 to position 1022 according to the numbering scheme of **FIG. 3** corresponds to the amino acid positions 202 to 461 according to the conventional numbering of the amino acid sequence of human TR-β which is publicly available. Also, the human TR-β LBD is expressed with a histidine tag, as described in Crowe *et al.*, *Methods in Molecular Biology* (1994) *31*:371-387, incorporated by reference herein.

The purification of human TR-β LBD is the same as that described above for the rat TR-α LBD with the following exceptions. First, before the purification step using the

hydrophobic interaction column, a step is added in which the expressed human TR-β LBD is purified using a nickel NTA column (commercially available from Qiagen, Chatsworth, CA) according to manufacturer's instructions, and eluted with 200 mM imidazole. The second difference is that in the purification of the human TR-β LBD, the purification step using a heparin column is omitted.

The crystallization of human TR-β LBD bound to T_3, Triac or GC-1 is as follows. Crystals are obtained in condition 7 of the factorial screen using hanging drops as before at ambient temperature (22°C) using the factorial crystallization screening trials of Jancarik & Kim (1991) and using the commercially available product from Hampton Research, Riverside). The following are optimum conditions: hexagonal bipyrimidal crystals are grown at 4°C for 2-3 days from hanging drops containing 1.0-1.2 M sodium acetate (pH unadjusted) and 0.1 M sodium cacodylate (pH 7.4), 3 mM DTT, with either a 1 μl protein solution, 1 μl precipitant solution or 2 μl protein solution, 1 μl precipitant solution and a 0.6 ml reservoir using silanized coverslip, at a protein concentration of 7-10 mg/ml. The best crystals have a limiting dimension of 200 μm. The following are optimum conditions for crystallization of the TR-β LBD with GC-1: hexagonal bipyrimidal crystals are grown at 4°C for 2-3 days from hanging drops containing 0.8-1.0M sodium acetate (pH unadjusted), 50-200nM sodium succinate, and 0.1M sodium cacodylate (pH 7.2), 3mM DTT, 1 μl protein solution, 1 μl precipitant solution and a 0.6ml reservoir using silanized coverslip, at a protein concentration of 7-10 mg/ml. The best crystals have a limiting dimension of 200μM. The unit cell dimensions are cell length a=b=68.73, cell length c=130.09. The unit cell angles are α=90°, β=90°, γ=120°.

The crystal system for human TR-β LBD bound to T_3, Triac or GC-1 is trigonal with the space group $p3_121$. The unit cell dimensions are cell length a = cell length b = 68.448 angstroms, cell length c = 130.559 angstroms. The angles are α = 90°, β = 90°, gamma = 120°.

EXAMPLE 9 - DETERMINATION OF LIGANDED TR-α LBD AND TR-β CRYSTAL STRUCTURES

Data from each cocrystal (Rat TR-α LBD with Dimit, T3 and IpBr2; Human TR-β LBD with Triac and GC-1) is measured on a Mar area detector at Stanford Synchrotron

Radiation Laboratory beamline 7-1 (λ = 1.08 angstroms) using 1.2° oscillations. Data from the cocrystal of the hTR-β LBD with Triac is measured on a Mar area detector at Stanford Synchrotron Radiations Laboratory beamline 7-1 (λ = 1.08 angstroms) using 1.0 oscillations. Data from the cocrystal of the hTR-β LBd with GC-1 is measured on a R-axis II area detector on a Rigaku rotating Cu anode (50kV, 300mA). The crystals are transferred into a cryosolvent containing 1.2M sodium acetate, 0.1M sodium cacodylate, adn 15% glycerol followed by a second transfer into 30% glycerol, then flash frozen in liquid nitrogen. An orientation matrix for each crystal is obtained using DENZO. The reflections are integrated with DENZO (commercially available from Molecular Structure Corp., The Woodlands, Texas) and are scaled with SCALEPACK (as described in Otwinowski, Z, *Proceedings of the CCP4 Study Weekend: "Data Collection and Processing,"* 56-62 (SERC Daresbury Laboratory, Warrington, UK 1993) incorporated by reference).

For rTR-α cocrystals, data from the T_3 cocrystal is measured with the b* axis approximately parallel with the spindle. The crystals are flash frozen at -178°C in a nitrogen gas stream with the MPD mother liquor serving as the cryosolvent. An orientation matrix for each crystal is determined using REFIX (Kabsch, W., *J. Appl. Crystallogr.* (1993) *26*:795-800 incorporated by reference). Reflections are integrated with DENZO, and are scaled with SCALEPACK.

For the T_3 data set, Bijvoet pairs are kept separate, and are locally scaled using MADSYS (W. Hendrickson (Columbia University) and W. Weis (Stanford University)).

Cocrystals prepared from the three isosteric ligands are isomorphous. MIR analysis is performed using programs from the CCP4 suite (Collaborative Computational Project, N.R. *Acta Crystallogr.* (1994) *D50*:760-763, incorporated by reference herein). Difference Pattersons is calculated for both T_3 and $IpBr_2$, taking the Dimit cocrystal as the parent. The positions of the three iodine atoms in the T_3 difference Patterson are unambiguously determined from the Harker section of the density map as peaks of 11σ above background. The positions for the two bromine atoms in the $IpBr_2$ cocrystals, are located independently, as peaks 8σ above the noise level. Phases for the LBD-122/410 are calculated from the solution to the $IpBr_2$ difference Patterson, and are used to confirm the location of the unique third iodine of the T_3 cocrystal. Halogen positions are refined with MLPHARE, including the anomalous contributions from the iodine atoms (Otwinowski, Z, *Proceedings of the CCPR*

Study Weekend 80-86 (SERC Daresbury Laboratory, Warrington, UK 1991)). The MIRAS phases are improved through solvent flattening/histogram matching using DM (Cowtan, K., *Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography* (1994) *31*: 34-38, incorporated by reference herein).

A model of the LBD-122/410 with Dimit bound is built with the program O from the solvent flattened MIRAS 2.5 angstrom electron density map (Jones *et al.*, *Acta Crystallogr.* (1991) A *47*:110-119, incorporated by reference herein). The initial model, without ligand, (Rcryst = 40.1%), is refined using least-squares protocols with XPLOR. The Dimit ligand is built into unambiguous Fo-Fc difference density during the following round. Subsequent refinement employs both least-squares and simulated annealing protocols with XPLOR (Brunger *et al.*, *Science* (1987) *235*:458-460), incorporated by reference herein). Individual atomic B-factors are refined isotropically. As defined in PROCHECK, all residues are in allowed main-chain torsion angle regions as described in Laskowski *et al.*, *J. Appl. Crystallogr.*, (1993) *26*:283-291, incorporated by reference herein. The current model is missing 34 residues (Met_{122}-Gln_{156}) at the N-terminus, and 5 residues (Glu_{406}-Val_{410}) at the C-terminus.

In addition, the following residues are not modeled beyond Cβ due to poor density: 184, 186, 190, 198, 206, 209, 240, 301, 330, 337, 340, 343, 359, and 395. The average B-value for protein atoms is 34.5 Å^2. The final model consists of the LBD-122/410, residues Arg_{157}-Ser_{183}, Trp_{185}-Gly_{197}, Ser_{199}-Asp_{206} and Asp_{208}-Phe_{405}; three cacodylate-modified cysteines: Cys_{334}, Cys_{380} and Cys_{392}; and 73 solvent molecules modeled as water (2003 atoms).

*$R_{sym} = 100 \times \sum_{hkl} \sum_i |I_i - I| / \sum_{hkl} \sum_i I_i$

†$R_{der} = 100 \times \sum_{hkl} |F_{PH} - F_H| / \sum_{hkl} |F_P|$

The occupancy for the two bromine sites is set to 35 electrons. The occupancies of the iodine sites are relative to this value.

§Phasing power = $\langle FH \rangle / \langle \epsilon \rangle$, where $\langle FH \rangle$ is the mean calculated heavy atom structure factor amplitude and $\langle \epsilon \rangle$ is the mean estimated lack of closure.

‖Rcullis = $\langle \epsilon \rangle / \langle iso \rangle$, where $\langle \epsilon \rangle$ is the mean estimated lack of closure and $\langle iso \rangle$ is the isomorphous difference.

¶Rcryst = 100 x $\sum_{hkl} |F_o - Fc| / \sum_{hkl} |F_o|$ where F_o and F_c are the observed and calculated structure factor amplitudes (for data $F/\sigma > 2$). The Rfree was calculated using 3% of the data, chosen randomly, and omitted from the refinement.

§ Correlation coefficient = $\sum_{hkl} (|F_o| - |F_o|) \times (|F_c| - |F_c|) / \sum_{hkl} (|F_o| - |F_o|)^2 \times \sum_{hkl} (|F_c| - |F_c|)^2$

EXAMPLE 10. PHASING OF THE rTR-α LBD AND hTR-β LBD COMPLEX WITH TRIAC

Due to the possible non-isomorphism of the rTRα LBD complex with Triac, a molecular replacement solution is determined using AMORE (Navaza, J., *Acta Crystallographica Section A-Fundamentals of Crystallography* (1994) *50*:157-63 from a starting model consisting of rTRα LBD complex with T_3, but with the ligand, all water molecules, and the following residues omitted: Asn 179, Arg228, Arg262, Arg266, and Ser 277. Strong peaks are obtained in both the rotation and translation searches, with no significant (> 0.5 times the top peak) false solutions observed (Table 6). Strong positive density present in both the anomalous and conventional difference Fourier maps confirm the solution. Maps are calculated using sigma-A weighted coefficients output by REFMAC (Murshudov, *et al.* "Application of Maximum Likelihood Refinements," in *Refinement of Protein Structures, Proceedings of Daresbury Study Weekend* (1996)) after 15 cycles of maximum likelihood refinement. Triac, the omitted residues, and water molecules 503, 504, 534 (following the numbering convention for the TR complex with T3) are built into the resulting difference density using O (Jones et. al.); the conformations of these residues are further confirmed in a simulated-annealing omit map (Brunger et. al.). The complete model is then refined using positional least-squares, simulated annealing, and restrained, grouped B factor refinement in XPLOR to an Rcryst of 23.6% and an Rfree of 24.1%

Phasing of a related LBD using the structure of the rTR-α LBD is conducted as follows. A molecular replacement solution for the hTR-β LBD complex with Triac is determined using AMORE from a starting model consisting of the rTR-α LBD complexed with T3, but with the ligand and all water molecules omitted. Strong peaks are obtained in both the rotation and translation searches, with no significant (>0.5 times the top peak) false solutions (Table 7). Strong positive density present in both the anomalous and conventional difference Fourier maps confirm the solution. Initial maps are calulated using sigma-A

weighted coefficients output by REFMAC after 9 cycles of maximum likelihood refinement. The real-space fit for each residues was calculated using OOPS (Kleywegt, GJ and Jones, TA, OOPS-a-daisy, ESF/CCP4 Newsletter 30, June 1994, pp. 20-24) and the residues with a real-space fit less than 2 standard deviations below the mean removed: Ala253-Lys263; Glu245-Leu250. To reduce bias, the following residues were modeled as alanine: Arg282, Arg316, Arg 320, Asn 331. Cycles of rebuilding and positional least-squares, simulated annealing, and restrained, grouped B factor refinement with XPLOR produce a model with an R_{cryst} of 25.3 and an R_{free} of 28.9%. The final model consists of hTR-β LBD residues Glu202-Gln252, Val264-Glu460; three cacodylate-modified cysteines with the cacodylate moeity modeled as free arsenic: Cys294, Cys298, Cys388, and Cys434; and 35 solvent molecules modeled as water.

EXAMPLE 11. CONNECTING QSAR WITH STRUCTURE IN THE THYROID HORMONE RECEPTOR

The conclusions of classic thyroid hormone receptor quantitative structure-activity relationships may be summarized as follows:

1) the R_4'-hydroxyl group functions as a hydrogen bond donor;

2) the amino-propionic acid interacts electrostatically through the carboxylate anion with a positively charged residue from the receptor;

3) the preferences of R_3/R_5 substituent are I>Br>Me>>H;

4) the preferences of the R_3'-substituent are Ipr>I>Br>Me>>H.

The structure of the thyroid hormone receptor ligand binding domain complexed with the agonists T3, $IpBr_2$, Dimit, Triac, and GC1 as provided herein, permits:

1) the identification of receptor determinants of binding at the level of the hydrogen bond;

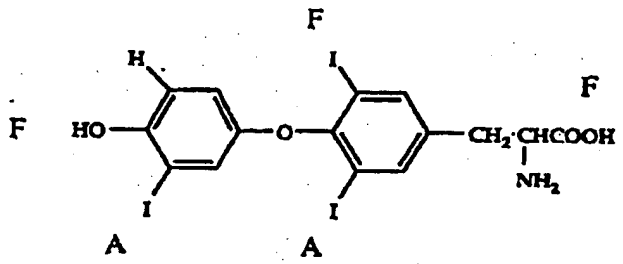
2) the association of these determinants with the predictions of classic thyroid hormone receptor QSAR; and

3) prediction as to which determinants of binding are rigid, and which are flexible, for both the ligand and the receptor.

This classification for the agonists of the type (R_1=amino-propionic, acetic acid; R_3,R_5=I,Br,Me; R_3'=Ipr,I) is given below (for the representative ligand T_3);

F = Fiducial (always satisfied)

A = Adjustable



Based upon the methods and data described herein, the following is an embodiment of the computational methods of the invention, which permit design of nuclear receptor ligands based upon interactions between the structure of the amino acid residues of the receptor LBD and the four different ligands described herein. The small molecule structures for the ligands can be obtained from Cambridge Structural Database (CSD), and three dimensional models can be constructed using the methods described throughout the specification. The following are factors to consider in designing synthetic ligands:

1) Histidine 381 acts as a hydrogen bond acceptor for the R_4' hydroxyl, with the optimal tautomer maintained by water molecules. See **FIG. 23 and FIG 24.** Histidine is the only hydrophilic residue in this hydrophobic pocket that surrounds the R_4' substituent. Histidine can be either a hydrogen bond acceptor or donor, depending on its tautomeric state. It is preferably a hydrogen bond donor, but can tolerate being a hydrogen bond acceptor, as for example, when there is a methoxy at the R_4' position of the ligand;

2) Arginines 228, 262, and 266 interact directly and through water-mediated hydrogen bonds with the R_1-substituent, with the electrostatic interaction provided by Arginine 266 (as in the Triac complex). This polar pocket is illustrated by **FIG. 23 - FIG. 25**. **FIG. 23** depicts T_3 in the TRα ligand binding cavity, where T3's amino-propionic R1-substituent interacts with Arg 228, HOH5O2, H9H5O3 and HOH5O4 via hydrogen bonds. **FIG. 24** depicts Triac in the ligand binding cavity, with its -COOH R_1 substituent in the polar pocket. In **FIG. 24**, Arg 228 no longer shares a hydrogen bond with the ligand, but the -COOH R_1 substituent forms hydrogen bonds with Arg 266. **FIG. 25** superimposes T_3

and Triac in the ligand binding cavity and shows several positionally unchanged amino acids and water molecules, and selected changed interacting amino acids and water molecules. The three figures illustrate parts of the polar pocket that can change and those parts that do not move upon binding of different ligands. For example, the Arg 262 at the top of the polar pocket does not move, even when the R_1 substituent has changed from a -COOH to an aminopropionic acid group. However, the other two Arginines, Arg 228 and Arg 266, demonstrate flexibility in the polar pocket to respond to the change in the size or chemical naure of the R_1 substituent.

3) Inner and outer pockets for the R_3/R_5 substituents are formed by Ser260, Ala263, Ile299; and Phe 218, Ile221, Ile222, respectively. See **FIGS. 21 and 22.** The inner pocket is filled by either the R_3 or the R_5 substituent, regardless of the size of the substituent, and may act as a binding determinant by positioning the ligand in the receptor. Optimally, the inner pocket amino acids interact with an R3 or R5 substituent that is no larger than an iodo group. If the inner pocket is filled by the R_3 substituent, then the outer pocket interacts with the R_5 substituent and vice versa. The outer pocket can adjust to the size of its substituent through main chain motion centered at the break in helix 3 (Lys220-Ile221), suggesting that the bending of H3, and motion of the N-terminal portion of H3, may represent a conformational change induced on ligand binding. The outer pocket has greater flexibility than does the inner pocket in terms of accommodating a larger substituent group.

4) A pocket for the R_3'-substituent is formed by Phe 215, Gly290, Met388. The pocket is incompletely filled by the R_3'-iodo substituent, and accommodates the slightly larger 3'-isopropyl substituent by movement of the flexible Met388 side chain and the H7/H8 loop. This pocket can accommodate R_3' substituents that are even larger than isopropyl, for example, a phenyl group.

The above information will facilitate the design of high affinity agonists and antagonists by improving automated QSAR methodologies and informing manual modeling of pharmaceutical lead compounds. For example, the inclusion of discrete water molecules provides a complete description of hydrogen bonding in the polar pocket for use with pharmacophore development: also, the identification of mobile and immobile residues within the receptor suggests physically reasonable constraints for use in molecular mechanics/dynamics calculations.

EXAMPLE 12. DESIGN OF AN INCREASED AFFINITY LIGAND

Direct interaction between the receptor and the ligand is limited in the polar pocket, which interacts with the R_1 substituent. While the lack of complementarity may contain implications for biological regulation, it also provides an opportunity for increasing affinity by optimizing the interaction between the amino acids of the polar pocket and the R_1 substituent of a synthetic ligand. The structure of the receptor-ligand interactions described herein enables design of an increased affinity synthetic ligand having two complementary modifications:

1) Remove the positively charged amine. The strongly positive electrostatic potential predicted for the polar pocket suggests that the positively charged amine of the aminopropionic acid R_1 substituent may be detrimental to binding. Suitable groups for substitution are suggested by the nature of nearby hydrogen bond partners: for example, Thr 275 O or Ser 277 N. See e.g. Tables in Appendix 2. For example, any any negatively charged substituent would be compatible for interacting with the amino acids of the polar pocket, including carboxylates, carbonyl, phosphonates, and sulfates, comprising 0 to 4 carbons. Another example of an R_1 substitution is an oxamic acid that replaces the amine of the naturally occurring ligand with one or more carbonyl groups.

2) Incorporate hydrogen bond acceptor and donor groups into the R_1-substituent to provide broader interactions with the polar pocket scaffold. Such hydrogen bond acceptor and donor groups incorporated into the R1-substituent will allow interactions that would otherwise occur with water molecules in the polar pocket. Specific waters include HOH 504 (hydrogen bonds with Ala 225 O and Arg 262 NH); and HOH 503 hydrogen bonds with Asn 179 OD1, Ala 180 N), both of which are present in all four complexes (TR LBD complexed with T3, TR LBD complexed with $IpBr_2$, TR LBD complexed with Dimit and TR LBD complexed with Triac). Analysis of the hydrogen bonding network in the polar pocket suggests replacement of HOH 504 with a hydrogen bond acceptor, and HOH 503 with an hydrogen bond donor (although the chemical nature of asparagine probably permits flexibility at this site). Thus, incorporating a hydrogen bond acceptor in an R1 substituent that could take the place of the HOH504 or incorporating a hydrogen bond acceptor in an R1 substituent that could positionally replace the HOH503, or a combination thereof, are methods of designing novel synthetic TR ligands.

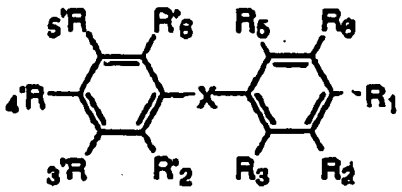
These two design approaches can be used separately or in combination to design synthetic ligands, including those in Table 5 (below).

A corollary to this approach is to design specific interactions to the residues Arg262 and Asn 179. The goal is to build in interactions to these residues by designing ligands that have R_1 substituents that form hydrogen bonds with water molecules or charged residues in the polar pocket.

High-affinity ligands also may be designed and selected using small molecules that bind to proximal subsites of the target nuclear hormone receptor that are identified in a structure-based screen and then linked together in their experimentally determined bound orientiations. Such a method has been described in design of high-affinity ligands for the FK506 binding protein (FKBP), stromelysin, gelatinase A, and human papillomavirus E2 (Hajduk *et al.*, *Science 278*:497-499 (1997)), which reference and its references are incorporated herein by reference. The preferred small molecules for screening are compounds of Formula I or derivatives thereof. For example, a compound of Formula I (ϕ-X-ϕ) or a derivative thereof (ϕ-X or X-ϕ) is screened for binding a target nuclear hormone receptor LBD. Proximal subsites of the nuclear hormone receptor include the hydrophobic and polar pockets of the LBD, and substites extended therefrom. As an example, Fourier transformation or nuclear magnetic resonance (NMR) -based structure screens can be used. When a NMR-based screen is used, binding can be detected from the amide chemical shift changes observed in two-dimensional heteronuclear single quantum correlation (HSQC) spectra aquired in the presence and absence of added compound. Once two ligands are identified that bind to the receptor, the crystal or solution structure of the ternary complex is determined. From the structural information, a compound is synthesized which links the two ligands, where the linker is selected based on structural information. The new compound is then screened for binding affinity, for example, using a binding assay as described herein. Only a few linked ligands need to synthesized and screened when using this approach.

Compounds of the invention also may be interatively designed from structural information of the compounds described above using other structure-based design/modeling techniques (Jackson, R.C., *Contributions of protein structure-based drug design to cancer chemotherapy. Semninars in Oncology*, 1997, *24*(2)L164-172; and Jones, T.R., *et al.*, *J. Med. Chem.*, 1996 *39*(4):904-917).

Table 5: Synthetic TR Ligands



R1	R2	R3	R5	R6	X	R'2	R'3	R'4	R'5	R'6
CO2H	H	Me	Me	H	O	H	Me	OH	Me	H
CH2CO2H		I	I		S		Et	SH	Et	
CH2CH2CO2H		Br	Br				nPr	NH2	nPr	
CH2CH(NH2)CO2H		Cl	Cl				iPr		iPr	
OCH2CO2H		Et	Et				Ph		nBu	
OCH2CH2CO2H		OH	OH				I		nPen	
NHCH2CO2H		NH2	NH2				Br		nHex	
NHCH2CH2CO2H		SH	SH				Cl		Ph	
CH2COCOCO2H									hetero	
									cycle	
NHCOCOCO2H									aryl	
COCO2H										
CF2CO2H										
COCH2CO2H										

Any combination of the above substituents in the biphenyl ether scaffold structure shown above may result in a potentially pharmacologically useful ligand for the thyroid hormone receptor. These novel ligands may be antagonists of the thyroid receptor.

TABLE 6: TR-α LBD-122/410

	Dimit	T3	$IpBr_2$	Triac
Data collection				
Cell dimensions				
a (Å)	117.16	117.19	117.18	118.19
b (Å)	80.52	80.20	80.12	81.37
c (Å)	63.21	63.23	63.13	63.73
β (°)	120.58	120.60	120.69	121.00
Resolution (Å)	2.2	2.0	2.1	2.45
Obs. Reflections, (no.)	57031	64424	66877	83573
Unique Reflections, (no.)	22327	21023	23966	18453
Completeness, (%)	87.0	82.4	93.7	96.0
*R_{sym} (%)	3.9	3.5	4.5	7.5
Phasing (15.0 - 2.5Å)				
†R_{der} (%)	-	19.6	11.6	
No. of sites	-	3	2	
‡Occupancy	-	44.6 (19.8)	35.0	
(Anomalous)	-	50.2 (23.7)	35.0	
		39.2 (22.3)		
§F_H/E				
centric (acentric)				
15.0-5.0 Å	-	3.67 (4.61)	2.25 (3.09)	
5.0-3.0 Å	-	2.23 (2.75)	1.25 (1.85)	
3.0-2.5 Å	-	1.64 (1.99)	1.15 (1.57)	
R_{Cullis}(%)				
15.0-5.0 Å	-	33	44	
5.0-3.0 Å	-	45	63	
3.0-2.5 Å	-	60	65	
Mean figure of merit	0.62	-	-	
MR Phasing (10-3.5Å)				
Rotation Search:				Θ_1 = 309.37
Euler Angles (°)				Θ_2 = 48.96
				Θ_3 = 127.28
§ correlation coefficient				34.3
Translation Search: Fractional coordinates				x = 0.1571
				y = 0.000

				z = 0.3421
§ correlation Coefficient				65.8
¶R factor				31.2
Refinement Resolution (Å)	15.0-2.2	5.0 - 2.0	15.0 - 2.2	25-2.5
$¶R_{cryst\ (\%)}$	20.5	22.1	21.4	23.6
$R_{free\ (\%)}$	22.7	24.0	22.4	24.1

TABLE 7: TR-β LBD-202/461

	Triac	T3	GC1
Data collection			
Space Group		P3121	
Cell dimensions			
a (Å)	68.9	68.45	68.73
c (Å)	131.5	130.56	130.09
Resolution (Å)	2.4	3.1	2.8
Obs. Reflections, (no.)	80196	55103	54104
Unique Reflections, (no.)	14277	6847	8987
Coverage (%)	97.0	95.7	97.1
$*R_{sym}$ (%)	5.1	4.6	5.5
MR Phasing (15.0 - 3.5Å)			
Rotation Search	Θ_1=39.13		
Euler Angles (°)	Θ_2=68.00		
	Θ_3=323.6		
§ correlation coefficient	21.6		
(Highest false peak)	(10.8)		
Translation Search	x=0.748		
Fractional Coordinates	y=0.158		
	z=0.167		
§ correlation coefficient	57.5		
(Highest false peak)	(38.7)		
	0.612		
*R factor	40.7	40.8	
Refinement			
Resolution (Å)	30-2.4		30-2.9
$¶R_{cryst\ (\%)}$	25.3		27.3
$R_{free\ (\%)}$	28.9		33.4

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The nuclear receptor ligands, particularly the TR ligands, of these references are herein incorporated by reference and can be optionally excluded from the claimed compounds with a proviso.

Headings and subheadings are presented only for the convenience of the reader and should not be used to construe the meaning of terms used within such headings and subheadings.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.